Facing uncertain diagnosis

The use of CSF biomarkers for the differential diagnosis of neurodegenerative diseases

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Facing uncertain diagnosis
The use of CSF biomarkers for the differential diagnosis of neurodegenerative diseases

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"Curiouser and curioser"

All quotes in this book are taken from the books *Alice's Adventures in Wonderland* and *Through the Looking Glass* by Lewis Carroll
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While difficulty walking can cause a severe limitation for many cases of Parkinson’s disease due to a symptom termed ‘freezing of gait’, many patients retain the ability to ride a bike.


Photo: Ineke riding her bike
Part 1

General Introduction

Alice: "When I get home I shall write a book about this place... If I ever do get home"
Chapter 1

General introduction and outline of the thesis

"Begin at the beginning," the King said, very gravely, "and go on till you come to the end: then stop."
Neurodegenerative Diseases

Neurodegenerative diseases are conditions affecting the cells of the central nervous system which result in the loss of cells, primarily neurons, in the brain and spinal cord. They are characterised by chronic, progressive degeneration of cells, in the motor, sensory, or cognitive systems of the brain. Depending on the brain area and extent of damage, this can bring about changes in memory, cognition, abstract thinking, skilled movement, behaviour, emotion, capacity for independent living and other disabilities. Most neurodegenerative diseases are chronic in nature and lead to the slow deterioration of cells over a period of many years. There are hundreds of different neurodegenerative diseases, the two most common of which are Alzheimer’s disease and Parkinson’s disease. Taken together, Alzheimer’s disease and Parkinson’s disease affect over 40 million people worldwide. Disturbingly, this estimate is expected to double over the next two decades as a result of increasing age in the world population and the fact that most neurodegenerative diseases affect people over the age of 55 years. Due to their chronic nature and the requirement for long-term health care, these diseases place a huge economic burden on society. Furthermore, gaps in healthcare provision mean that family members and friends often take on the burden of caring for their loved ones creating an additional social burden.

Among the neurodegenerative diseases, many share similar clinical features, a fact that is perhaps not unexpected given the partial overlap in underlying neurodegenerative processes and extent of the associated pathology. Hence, neurodegenerative diseases are often categorised according to their shared clinical features leading to classifications such as ‘Dementia disorders,’ ‘Movement disorders,’ or ‘demyelinating disorders.’ Even within these categories, there are frequent multiple diseases sharing similar clinical features that make it difficult for clinicians to distinguish one disease from another. Dementia disorders, for example, all share features of altered memory and/or cognition, behaviour, emotion, and an inability to follow simple instructions that interfere with daily functioning. Similarly, movement disorders involve altered voluntary and involuntary movements, often associated with a tremor, loss of balance or muscle weakness. Furthermore, many of the key features that can distinguish one disease from another appear at much later stages of disease, making an accurate diagnosis at early stages of disease challenging for clinicians. What’s more, there is sometimes an overlap in clinical features between the classification groups. For example, Dementia with Lewy bodies presents with Parkinson’s disease-like movement difficulties, termed parkinsonisms, together with memory loss, behavioural disturbances, hallucinations, confusion, sleep disorder associated with dreaming, and trouble interpreting visual information. Similarly, dementia can develop during the course of Parkinson’s disease, but the dementia often occurs 10 to 15 years after parkinsonisms appears rather than at the same time. Finally, two diseases can co-occur so that, for example, one patient may have both Parkinson’s disease and Alzheimer’s type dementia. Accordingly, many patients will be left facing an uncertain diagnosis.
On the surface it might seem inconsequential to give separate labels to diseases with such similar clinical features. However, there are actually many reasons why a precise diagnosis might be important. One of the most critical reasons for wanting an accurate diagnosis is to ensure that optimal medical treatment is provided. Unfortunately there are very few medicines currently available for the treatment of symptoms of neurodegenerative diseases, and none that can cure them. Crucially, the use of a particular medication for one disease may be inadvisable for another disease due to known side-effects, which are occasionally fatal. For example, neuroleptics may be used in patients with Alzheimer disease, but should be avoided in patients with dementia with Lewy bodies. Other judicious reasons for aspiring to a precise diagnosis include: assignment of patients to appropriate patient groups for facilitating the determination of treatment effects in those groups when testing trial medications; prediction of disease outcome, including alertness to specific and sometimes life-threatening complications (e.g. development of nocturnal stridor in patients with multiple system atrophy); and to facilitate examination of the pathological and neuroanatomical features of individual diseases. At other times, an accurate diagnosis of disease is desirable simply because it is of comfort to patients to put a name to their disease.

Differential Diagnosis of Neurodegenerative Diseases

This brings me to the main question underlying this thesis: “How can we improve the differential diagnosis of neurodegenerative diseases?” That is, how can we better distinguish one neurodegenerative disease from another? In trying to answer this question, a lot of prior research has focussed on searching for suitable biological markers, or ‘biomarkers’. A biomarker is essentially any biological indicator used to identify the presence of a disease. The most common biomarkers used in current medicine involve blood tests that measure abnormal levels of proteins or other substances in the blood. For example, high levels of the troponin proteins and the enzyme, creatine kinase can be used as blood biomarkers for diagnosis of cardiac injury following a myocardial infarction (heart attack). However, biomarkers are not just found in blood. Measurement of proteins can also be performed in other biological fluids such as urine, synovial fluid of the joints, pleural fluids surrounding the lungs, or cerebrospinal fluid (CSF) which surrounds the brain and spinal cord. Furthermore, biomarkers can be derived from imaging techniques such as computer tomography or magnetic resonance imaging (to look at changes in brain volume and neuronal loss), from positron emission tomography with 18F-2-deoxy-2-fluoro-D-glucose or Pittsburgh compound B, which can be used in positron emission tomography (to provide information about cerebral amyloid beta accumulation), or from dopamine transporter single-photon emission computed tomography (to visualise dopaminergic degeneration in Parkinson's disease and multiple system atrophy).

In terms of biomarkers for neurodegenerative diseases, if we could accurately measure brain-specific proteins in biological fluids we might expect that any clear differences observed between healthy and disease subjects would be an indication of pathological changes in the brains of the diseased subjects. Although blood-based biomarkers are highly desirable for disease diagnosis because they are relatively easy to obtain, cause
minimal discomfort for the patient and are cost- and time-efficient, blood as a source of brain-specific biomarkers has drawbacks. Blood contains huge numbers of proteins owing to the fact that there is extensive exchange of proteins and other substances between the blood and all organs of the body during blood circulation, not just the brain and spinal cord. Furthermore, the exchange of proteins between the central nervous system and periphery is highly regulated by the blood-brain and blood-CSF barriers meaning that brain-specific proteins are present in the blood at very low concentrations making it extremely difficult to detect changes in brain-specific proteins in the blood. This is why the focus of current research has moved towards examining the potential of cerebrospinal fluid (CSF) biomarkers in the differential diagnosis of disease.

**Why focus on cerebrospinal fluid biomarkers?**

CSF surrounds the brain and spinal cord and represents the primary component of the brain extracellular matrix that exchanges a broad array of biological products with the central nervous system. Thus the CSF matrix contains a complex mixture of proteins and other biological substances that can reflect dynamic changes occurring in the physiological and pathological state of the brain. Furthermore, it can be accessed fairly readily using lumbar puncture (Figure 1). Of relevance to biomarker research, many of these proteins are brain specific and the exchange of proteins between the brain and the CSF and the CSF and blood is tightly controlled by the blood-brain and blood-CSF barriers (Figure 2). Hence, levels of these proteins in CSF largely reflect physiology that is specific to the central nervous system. Thus aberrant concentrations of brain-specific proteins in CSF are likely to reflect underlying disease pathology.
Figure 1. Lumbar puncture. During a lumbar puncture (or spinal tap), a needle is carefully inserted into the lower (lumbar) part of the spine and a small sample of CSF is removed for testing. Reprinted with permission from Ref 35 © (2006) American Medical Association.
Fig. 2. Barriers of the brain. Barriers of the brain are present at three main sites: the brain endothelium forming the blood–brain barrier (BBB) (1), the arachnoid barrier cell layer (2) forming the middle layer of the meninges, and the blood-CSF barrier (3) located at choroid plexus epithelium, which secretes cerebrospinal fluid (CSF). At each site, the physical barrier is caused by tight junctions that reduce the permeability of the paracellular (intercellular cleft) pathway. Printed, with permission, from Refs 35,36 © 1990 (Kluwer Academics).
Since the cerebrospinal fluid contains many thousands of different components, of which there are thousands of different proteins, it may seem a daunting task to identify just a few proteins that might be of interest for measurement as potential biomarkers. Many clues are already available from studies which have examined altered proteins in familial diseases, that is, those in which a genetic alteration leads to disease. While most neurodegenerative diseases are sporadic (occur randomly), a minority have an underlying genetic component. Determining which proteins are encoded by these genes, and thus altered in familial disease, also provides a starting point for similar investigations in sporadic form(s) of disease. For example, the α-synuclein (α-Syn) gene is frequently mutated in familial forms of Parkinson’s disease leading to loss of function of the protein α-Syn. This discovery led to other studies investigating the structure and function of α-Syn, and the assessment of CSF α-Syn levels in sporadic forms of disease compared with controls. These studies have shown that α-Syn is also altered in sporadic forms of disease. However, levels of α-Syn in CSF have not yet adequately been shown to distinguish Parkinson’s disease from healthy subjects or from other neurodegenerative diseases.

Other methods being used to identify proteins of interest for biomarker research include: genome-wide association studies – a broader study identifying gene mutations involved in neurodegenerative disease; proteomics – examination of proteins; and metabolomics – the study of metabolites. These methods have identified a large and diverse range of proteins that are altered, or likely to be altered, in neurodegenerative diseases. Indeed many of the proteins analysed during the research performed for this thesis have been identified by earlier studies investigating the underlying molecular mechanisms of these diseases using various methods.

Molecular mechanisms of disease and biomarker identification

Protein misfolding and aggregation

One of the most comprehensively investigated mechanisms of pathology in neurodegenerative diseases is that of protein misfolding and aggregation. These investigations originated with Alois Alzheimer who, in 1906, was the first person to describe primary neuropathological features in the brain of a woman with slowly progressing dementia. He identified deposits of the protein amyloid beta (Aβ) and degenerating neurons with bundles of fibrils (neurofibrillary tangles) scattered in the brain and his reports ultimately gave rise to the name ‘Alzheimer’s dementia’. This led to subsequent investigations of the levels of Aβ, tau and phosphorylated tau in the CSF of Alzheimer’s patients compared with controls. These studies have consistently shown altered levels of these proteins in patients with Alzheimer’s disease compared with healthy subjects. Alzheimer’s disease patients have much lower levels of CSF Aβ and higher levels of total and phosphorylated tau than healthy controls. Since these markers show clear differences between Alzheimer’s patients and healthy controls, they are increasingly being used as an adjunct to clinical diagnosis.
Not only Alzheimer’s disease but many other neurodegenerative disease such as amyotrophic lateral sclerosis, Huntington’s disease, Parkinson’s disease and multiple system atrophy can be characterised by hallmark pathological features visible at post-mortem (after death) examination of the brain. Most of these features include degeneration of specific brain regions and deposits of abnormal proteins that are thought to be specific to individual diseases. Some of the best-described of these brain changes include: the presence of α-syn containing Lewy bodies and loss of dopamine-producing neurons in the striatum of Parkinson’s disease; α-syn containing glial inclusion bodies in multiple system atrophy; deposits of TDP-43 in amyotrophic lateral sclerosis and frontotemporal dementia, and aggregates of the protein huntingtin in Huntington’s disease. In other neurodegenerative diseases, such as the demyelinating disease multiple sclerosis, changes in the white matter of the brain and spinal cord can be seen using special imaging techniques such as magnetic resonance imaging and computer tomography rather than being characterised by abnormal protein deposits. However, more recently, the discovery of aggregated fibronectin in demyelinated lesions in the white matter of brains from multiple sclerosis patients suggesting that protein aggregates may also play a role in, or arise from, the pathogenesis of demyelinating diseases.

Most proteins associated with neurodegeneration are natively unfolded and initiation of protein misfolding in particular cells likely facilitates the aggregation and accumulation of the specific protein. Although the underlying mechanisms of protein misfolding have not been fully elucidated, another common feature of many neurodegenerative diseases, ‘oxidative stress’, is thought to facilitate the aggregation and accumulation of proteins through post-translational, covalent modifications of the proteins.

Oxidative stress

The brain is a highly aerobic organ, consuming more oxygen per weight than any other organ in the body and leading to the generation of reactive oxygen species and reactive nitrogen species, which can have damaging effects on cells. Oxidative stress arises due to an imbalance between production of reactive oxygen species and/or dysfunction of antioxidant protection systems and is a common feature of neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and multiple sclerosis. Oxidative stress can be identified by measures of lipid and DNA peroxidation and protein carbonyl moieties, which are increased in neurodegenerative disease states compared with healthy controls. Moreover, the proteins that are oxidised appear to be specific to individual diseases. In Alzheimer’s disease, specifically oxidised proteins include glutamine synthetase, α-enolase, amyloid-β, and the prolyl peptidyl isomerase, Pin1 whereas α-synuclein and DJ-1 are prone to oxidation in Parkinson’s disease and β-actin, glial fibrillary acid protein and neurofilaments are the target of protein carbonylation in multiple sclerosis. Since carbonylation can easily be detected in experimental designs, measurement of carbonylated proteins is a common measurement of oxidative stress.
Neurotransmitter dysfunction

In addition to oxidative stress, dysfunction in neurotransmitter transmission is a common element of neurodegeneration. The loss of dopaminergic transmission arising from death of dopaminergic neurons of the substantia nigra is well-recognised in Parkinson’s disease and is known to cause some of the more characteristic motor dysfunctions such as resting tremor and bradykinesia. Similarly, alterations in cholinergic transmission, namely loss of cholinergic neurons and decreased acetylcholinesterase activity, are thought to underlie many of the cognitive and behavioural symptoms of Alzheimer’s disease and dementia in Parkinson’s disease since acetylcholine is a neurotransmitter essential for processing memory and learning. Indeed, many of the medications available to treat these symptoms are targeted at counteracting the loss of acetylcholinesterase activity.

More recent investigations have highlighted the likelihood that dysfunction of other neurotransmitters, particularly glutamate, but also epinephrine, norepinephrine and serotonin, and their metabolites are involved in the non-motor deficits of movement disorders and many of the behavioural deficits observed in dementia disorders. Glutamate is the predominant excitatory neurotransmitter in the human central nervous system and is normally kept at relatively low concentrations in the extracellular spaces of the brain. However, in Alzheimer’s disease in particular, chronic low-level chronic stimulation of glutamate receptors has been associated with glutamate excitotoxicity leading to overstimulation of neuronal signalling and eventual neuronal death. Disturbances in glutamate transmission are not confined to Alzheimer’s disease but are implicated in several other neurodegenerative diseases including Parkinson’s disease, amyotrophic lateral sclerosis and multiple sclerosis.

Where our quest begins...

Taken together, the investigation of protein misfolding and aggregation, oxidative stress, and neurotransmitter alterations have identified a plethora of potential biomarkers of individual neurodegenerative diseases. This raises the question “Where do we start?” Alzheimer’s disease is the most common and most studied of all neurodegenerative diseases. Furthermore, several biomarkers have already been established to aid in the diagnosis of Alzheimer’s disease. Thus it makes sense to begin by attempting to build on the available knowledge. Hence our investigation began with how we can improve the diagnosis of Alzheimer’s disease and improve the differential diagnosis of the various forms of dementia using a combination of the so-called ‘signature biomarkers’ of Alzheimer’s disease (Aβ-42, total tau and Phosphorylated tau) combined with more recently identified proteins showing promise as biomarkers. We then continued our journey by exploring possible CSF biomarkers for the differential diagnosis of the second most common neurodegenerative disorder, Parkinson’s disease, from analogous diseases. Sporadic forms of Alzheimer’s disease and Parkinson’s disease are most commonly associated with aging and predominantly affect people over the age of 55. Since neurodegenerative diseases are not confined to elderly patients, we also began an initial exploration into the use of CSF biomarkers for the differential diagnosis of the most common neurodegenerative
disease among younger adults, multiple sclerosis. The rationale for the choice of target proteins and literature supporting those choices, are outlined in the individual chapters.

In summary, neurodegenerative diseases affect vast numbers of people every year. Despite decades of intensive research, precise diagnosis of many individual diseases continues to challenge clinicians. Distinguishing one neurodegenerative disease from another can be rather difficult when using clinical symptoms alone, certainly in early disease stages when the clinical picture is often still incomplete. Moreover, in the early phases of disease, many neurodegenerative diseases share common symptoms, the rate of disease progression is not yet apparent, and the response to medical treatment is often not yet fully clear. Therefore, additional tools to help clinicians in the differential diagnosis of neurodegenerative diseases are needed. The current challenge for researchers is to identify potential biomarkers enabling earlier, faster and more accurate diagnosis of specific neurodegenerative diseases.

**Aims of the thesis**

The primary aim of this thesis was to determine the diagnostic utility of recently identified potential CSF biomarkers, and combinations of these biomarkers, as diagnostic tools in the differential diagnosis of dementias, movement disorders, and demyelinating diseases.

**Outline of the thesis**

**Part one | General introduction**

Chapter one, this introduction, acquaints the reader with the neurodegenerative diseases, their molecular and pathological features, the importance of differential diagnosis of disease, and the usefulness of CSF biomarkers in differential diagnosis. Finally, the aims and outline of the thesis are provided.

**Part two | CSF biomarkers in the differential diagnosis of dementia disorders**

Chapter two probes the diagnostic value of combining measures of MHPG with the traditional biomarkers for Alzheimer’s disease (Aβ, p-tau and t-tau) for improving the differential diagnosis of dementia with Lewy bodies from other forms of dementia such as Alzheimer’s disease, vascular dementia and fronto-temporal dementia

Chapter three outlines the development of an enzyme-linked immunoassay for the specific measurement of oxidised Pin1 in brain tissue. This assay can be used to measure levels of oxidised Pin1 in brain tissue and was used to compare levels of oxidised Pin1 in the brain tissue of Alzheimer’s disease compared with that of control tissue.
Part three | CSF biomarkers in the differential diagnosis of movement disorders

Chapter four explores levels of the CSF proteins neurofilament light chain, fms-like tyrosine kinase ligand (FLT3L), and total tau in the differential diagnosis of Parkinson’s disease from multiple system atrophy and compares levels of these proteins in non-neurological controls.

Chapter five examines the diagnostic utility of the Parkinson’s disease-linked proteins, DJ-1, as a biomarker for differentiating Parkinson’s disease from multiple system atrophy and for comparison with controls.

Chapter six investigates the levels of neurotransmitter metabolites in the cerebrospinal fluid of patients with vascular parkinsonism compared with Parkinson’s disease patients and illuminates possible reasons why patients with vascular parkinsonism might respond more poorly to levodopa medications than those with Parkinson’s disease.

Part four | CSF biomarkers in the differential diagnosis of demyelinating disorders

Chapter seven outlines the development and optimisation of enzyme-linked immunoassays for the measurement of glutamine synthetase and myelin basic protein in cerebrospinal fluid as possible disease biomarkers. In addition this chapter highlights the sometimes difficult task of detecting proteins and how obstacles might be overcome by using modifications of standard assays.

Chapter eight studies changes in levels of glutamine synthetase in the cerebrospinal fluid of patients with demyelinating disorders (multiple sclerosis, neuromyelitis optica, optic neuritis) compared with controls. The potential use of glutamine synthetase as a biomarker is discussed.

Part five | General Discussion and Summary

Chapter nine provides a general discussion of the findings and conclusions made from the findings outlined in the thesis, and identifies future perspectives for the studied biomarkers.

Chapter ten summarises the research findings and their relevance.
References


Chapter 1


Photo series - Part 2: A key feature of Alzheimer's disease is progressive loss of memory, with most recent memories being lost first.
Part 2

The use of CSF biomarkers for the differential diagnosis of dementia

“It's a poor sort of memory that only works backwards” says the White Queen to Alice.
Chapter 2

Addition of MHPG to Alzheimer’s disease biomarkers improves differentiation of dementia with Lewy bodies from Alzheimer’s disease but not other dementias

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Alzheimer’s & Dementia 2013, In press

"I can't go back to yesterday - because I was a different person then."
Abstract

Background: Overlapping clinical features make it difficult to distinguish dementia with Lewy bodies (DLB) from Alzheimer’s disease (AD) and other dementia types. In this study we aimed to determine whether the combination of cerebrospinal fluid (CSF) biomarkers, amyloid-β_42 (Aβ_42), total tau protein (t-tau) and phosphorylated tau (p-tau), in combination with 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), could be useful in discriminating DLB from vascular dementia (VaD) and frontotemporal dementia (FTD), as we previously demonstrated for differentiation of DLB from AD.

Methods: We retrospectively analysed concentrations of MHPG, Aβ_42, t-tau, and p-tau in CSF in patients with DLB, AD, VaD, and FTD. Using previously developed multivariate logistic regression models we assessed the diagnostic value of these CSF parameters.

Results: The currently used combinations of Aβ_42, t-tau, and p-tau yielded a sensitivity of 61.9% and a specificity of 91.7% for the discrimination between DLB and AD, but could not discriminate between DLB and VaD or FTD. The addition of MHPG to Aβ_42, t-tau, and p-tau improves the discrimination of DLB from AD, yielding a sensitivity of 65.1% and specificity of 100%, but could not distinguish DLB from other forms of dementia.

Conclusions: Our results confirm, in a separate patient cohort, that the addition of MHPG to Aβ_42, t-tau, and p-tau improves the discrimination of DLB from AD but not the differentiation of DLB from VaD or FTD.
Introduction

The number of people living to older age is increasing globally. Advancing age is the single biggest risk factor for the development of dementia and thus the prevalence of dementia is also rapidly increasing and is fast becoming a major economic and health burden. Up to 9.4% of the European population aged ≥65 years are affected by dementia and this estimate is expected to double by the year 2040. After Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and vascular dementia (VaD) are the second most common forms of dementia.

DLB patients often present with varying combinations of cognitive, behavioural, and physical symptoms that make differential diagnosis challenging. Not only is there a considerable overlap of clinical features among the dementias, but patients also often have a mixed pathology. Clinically, DLB is characterised by fluctuating cognition, visual hallucinations, and agitation. Such behavioural symptoms are often treated with neuroleptics, but these medications are contraindicated in DLB as neuroleptic sensitivity can lead to worsening of symptoms or even sudden death in some DLB patients. An earlier study indicated that behavioural disturbances in DLB patients could be improved using treatment with the cholinesterase inhibitor rivastigmine. However, more recent reviews indicate that there was variable or no positive response of cholinesterase inhibitors on cognitive function.

A timely diagnosis of DLB is necessary to ensure optimal management of a patient, but this is often difficult to accomplish within the patient's lifetime. Given that AD and DLB differ significantly in aspects such as memory, behaviour, and sleep patterns, an earlier diagnosis would aid in early recognition of disease-specific symptoms, clinical decision-making, adequate patient counselling, and targeted treatment strategies. Thus, biomarkers capable of affording more precise differentiation between DLB and other forms of dementia would be of tremendous clinical and diagnostic value.

In a previous study, we assessed which combination of cerebrospinal fluid (CSF) biomarkers could provide optimal differentiation of DLB from AD. We first assessed concentrations of Amyloid-β (Aβ), total tau protein (t-tau) and phosphorylated tau (p-tau), the traditionally used biomarkers for AD, for their efficacy in differentiating between AD and DLB. In addition, we tested concentrations of 3-methoxy-4-hydroxyphenylethylenglycol (MHPG), the primary metabolite of the neurotransmitter norepinephrine. We observed significantly reduced levels of MHPG in the CSF of DLB patients compared with AD patients and, when MHPG concentrations were used in combination with Aβ, t-tau, and p-tau levels in CSF, we could discriminate DLB from AD with enhanced diagnostic accuracy.

Reduced MHPG concentration in the CSF of DLB patients is congruent with earlier observations of decreased concentrations of norepinephrine in the putamen and neocortex, and substantial degeneration of locus coeruleus noradrenergic neurons in the brains of patients with DLB. Moreover, concentrations of MHPG are generally unchanged or only slightly reduced in VaD and FTD, whereas they are generally unchanged or increased in AD compared with controls. Thus significant reductions in CSF MHPG may represent a unique feature of DLB. Therefore, in the current study we...
aimed to confirm, in a separate patient cohort, that the addition of MHPG to Aβ 42, t-tau, and p-tau in CSF improves the discrimination of DLB from AD, and we expanded our earlier study to determine whether these CSF parameters could also be useful in discriminating DLB from VaD and FTD.

Methods

Patients

CSF samples were collected according to standard protocols and all patients or their legal representative provided informed consent for lumbar puncture. We identified patients with a clinical diagnosis of DLB, AD, VaD, or FTD using up-to-date databases of patients who had been referred to either the movement disorders clinic of the Department of Neurology of the memory clinic of the Department of Geriatric Medicine at the Radboud University Medical Centre during the period May 1996 to December 2009. Patients for whom lumbar puncture was performed as part of the diagnostic work-up and for whom informed consent was obtained, were included in the study. From these groups we selected patients for whom relevant CSF parameters were available for inclusion in the current study (Figure 1), but we excluded those patient who were part of our earlier study.

As part of the referral process, patient diagnosis was made based on a detailed medical history, comprehensive physical and neurologic examination, routine laboratory testing, and a brain magnetic resonance imaging (MRI) scan. Diagnoses were established by a multidisciplinary team consisting of a geriatrician, a neurologist, and a neuropsychologist. Clinical diagnosis was confirmed according to the consensus criteria for DLB, AD, VaD, and FTD. In July 2012, a geriatrician and memory clinic specialist (JAHRC) reassessed the final clinical diagnosis by clinical chart review to improve diagnostic accuracy. Patients with FTD were also divided into subtypes according to newer clinical criteria for FTD by the same geriatrician in February 2013.

CSF parameters

CSF samples were obtained by lumbar puncture and collected in polypropylene tubes and centrifuged for 5 minutes (860 g at room temperature). Samples were then transferred to clean polypropylene tubes and stored at -80°C. All CSF measurements were performed by technicians trained in CSF analysis. These technicians were neither aware of the clinical diagnosis nor of the results of other individual tests at the time of CSF analysis. The CSF measures taken into consideration for this study were MHPG, Aβ 42, t-tau, and p-tau. MHPG measurements were performed within 4 weeks of CSF collection in all but 8 cases. These final 8 cases were measured for the purpose of this study and been stored at -80°C until the time of measurement. The method of analysis and the validation of MHPG have been described previously. The inter-assay coefficient of variation (CV) for MHPG was 4.8%.

Aβ 42, t-tau, and p-tau were measured using commercially available kits (INNOTEST®, Innogenetics N.V., Ghent, Belgium). All assays were performed according to the manufacturer’s instructions and the methodology did not change during the period in
Biomarkers distinguish Alzheimer’s disease from dementia with Lewy bodies

which the analyses were performed. For the measurement of t-tau we included additional standards with concentrations of 1200 pg/mL and 2400 pg/mL. Internal controls were included in all enzyme-linked immunosorbent assays (ELISAs) to control for inter-assay variation. Inter-assay CVs were 2.9% - 7.0% for Aβ_{42}, 1.7- 6.0% for t-tau and <5.0% for p-tau. Measurements of Aβ_{42}, t-tau, and p-tau were performed within 4 weeks of collection for all samples received during or after 2004 and were performed retrospectively for all CSF samples obtained prior to 2004. All patient information was decoded to maintain patient confidentiality.

Statistical analysis

Statistical analyses were carried out using GraphPad PRISM, version 5 (GraphPad, Inc., San Diego, CA), and SPSS software, version 16 (SPSS, Inc. Chicago, IL). Normally distributed data were analysed using Student's t-tests for comparison between two groups and one-way analysis of variance (ANOVA) with Tukey's post hoc analyses to determine between-group differences in the case of multiple groups. In the case of non-Gaussian-distributed CSF parameters, data were log-transformed prior to analysis or, alternatively, analysed using the Mann-Whitney U-test. Receiver operator curve (ROC) analysis was employed to evaluate the diagnostic value of CSF parameters and the previously developed models. ROC curve comparisons were performed using MedCalc version 12.2.1 (MedCalc Software, Belgium).
Results

Patients

At the time of selection, 14 patients fulfilled the criteria for DLB, 15 for VaD, 26 for FTD, and 64 for AD. Patients' demographics and CSF parameters are presented in Table 1. Given the retrospective nature of this study, Mini-Mental State Examination (MMSE) findings and disease duration were not available for all patients, particularly for the DLB group. However, MMSE and disease duration were not correlated with any of the CSF parameters in any of the patient or control groups. The average age at time of lumbar puncture for the FTD patients was significantly lower ($p < .001$) than for all other patient groups, but no significant differences in age were apparent for the AD, DLB, or VaD groups.

CSF parameters

As depicted in Figure 2, CSF analyses showed reduced levels of $\text{Aβ}_{42}$ for AD and DLB, increased levels of t-tau and p-tau for AD, and decreased levels of MHPG for DLB. Repeat analyses adjusting for age, gender, disease duration, and cognitive function did not markedly alter our results. We observed some unexpected heterogeneity in all CSF parameters for the AD patients. However, compared with our reference values, we observed an expected decrease in $\text{Aβ}_{42}$, and increase in t-tau in our AD patients that was much greater than for all other groups, suggesting that these patients were correctly classified as AD. The percentages of patients with decreased CSF $\text{Aβ}_{42} \ (\leq 500 \text{ ng/L})$ were as follows: AD, 70.3%; DLB, 50%; VaD, 15.4%; and FTD, 11.5%. CSF t-tau was increased ($\geq 350 \text{ ng/L}$) in AD patients (78.1%) and less frequently in VaD (46.2%), FTD (46.2%), and DLB (28.6%) patients. p-tau was increased ($\geq 85 \text{ ng/L}$) in most AD patients (62.5%) and in smaller numbers of FTD (23.1%), VaD (15.4%), and DLB (7.1%) patients. Finally, MHPG was decreased ($<38 \text{ nmol/L}$) in 50% of DLB patients compared with 19.2% of FTD patients, 15.4% of VaD patients, and 14.1% of AD patients.

We also observed heterogeneity in the CSF parameters for our FTD patients, which we further investigated by analysing differences between different FTD subtypes: behavioural variant FTD (bvFTD, $n=7$); primary progressive aphasias of the semantic/agrammatic type (sdFTD, $n=3$) and nonfluent type (nfFTD, $n=9$); FTD with motor neuron disease (FTD-MND, $n=2$); and FTD with parkinsonism ($n=3$). Two patients were excluded from the subtype analysis as we had insufficient information to classify these 2 patients into subtypes. We found no significant differences in $\text{Aβ}_{42}$, t-tau, and p-tau between FTD subtypes (refer to Table S1 in supplementary data). Interestingly, we observed that all FTD subgroups, except bvFTD, had MHPG levels similar to those of the DLB group.

Statistical analysis revealed that $\text{Aβ}_{42}$ levels were significantly lower in the AD group than in the VaD ($p < .05$) and FTD ($p < .001$) groups, but were similarly decreased in DLB patients. $\text{Aβ}_{42}$ levels were also significantly lower in the DLB group than the FTD group ($p < .05$). Most of this difference in $\text{Aβ}_{42}$ levels between FTD and AD was accounted for the nfFTD ($p < .001$) group and bvFTD group ($p < .05$) subtypes. t-tau levels were significantly higher for the AD group compared with the DLB and VaD groups (both $p < .001$), but no further between group differences were found. Levels of p-tau were increased significantly in
Table 1

Characteristics of the diagnostic groups

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>DLB</th>
<th>AD</th>
<th>VaD</th>
<th>FTD</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>14</td>
<td>64</td>
<td>15</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age, years (SD)*</td>
<td>72.4 (8.0)</td>
<td>73.1 (8.3)</td>
<td>76.5 (4.8)</td>
<td>61.6 (8.4)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gender, M/F (% male)</td>
<td>10/4 (71%)</td>
<td>13/5 (20%)</td>
<td>10/5 (67%)</td>
<td>17/9 (65%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MMSE, average (s.d.)</td>
<td>22 (5.0), n = 4</td>
<td>20 (4.0), n = 61</td>
<td>18 (3.7), n = 12</td>
<td>18 (7.3), n = 12</td>
<td>0.463</td>
</tr>
<tr>
<td>Disease duration, months (s.d.)</td>
<td>24 (24.0), n = 6</td>
<td>15 (15.6), n = 61</td>
<td>17 (15), n = 12</td>
<td>7.3 (14), n = 10</td>
<td>0.112</td>
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<tr>
<td>CSF parameters ‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHPG nmol/L (s.d.)</td>
<td>38.9 (12.6)</td>
<td>50.0 (12.8)</td>
<td>49.9 (15.2)</td>
<td>46.5 (10.9)</td>
<td></td>
</tr>
<tr>
<td>Aβ42, ng/L</td>
<td>513 (154)</td>
<td>481 (145)</td>
<td>568 (168)</td>
<td>683 (215)</td>
<td></td>
</tr>
<tr>
<td>t-tau, ng/L</td>
<td>287 (159)</td>
<td>727 (484)</td>
<td>380 (269)</td>
<td>443 (303)</td>
<td></td>
</tr>
<tr>
<td>p-tau, ng/L**</td>
<td>58 (17)</td>
<td>112 (53)</td>
<td>54 (19)</td>
<td>64 (31)</td>
<td></td>
</tr>
<tr>
<td>t-tau/ Aβ42 ratio</td>
<td>1.72 (1.51)</td>
<td>0.62 (0.39)</td>
<td>0.77 (0.80)</td>
<td>0.72 (0.62)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DLB, dementia with Lewy bodies; AD, Alzheimer’s disease; VaD, vascular dementia; FTD, frontotemporal dementia; SD, standard deviation; M, males; F, females; CSF, cerebrospinal fluid; MHPG, 3-methoxy-4-hydroxyphenylethanol.  
* At time of lumbar puncture; † p-values are presented for differences using one-way analysis of variance in the case of Gaussian-distributed data or Kruskal-Wallis in the case of non-Gaussian-distributed data (disease duration only). Gender distribution was analysed using the chi-square test.  
‡ Analysis of variance of transformed data was performed to test differences in MHPG, t-tau, and p-tau and the Kruskal-Wallis test was used to test differences in Aβ42 for each group compared with DLB (p < 0.1 indicates a trend).  
§ p < 0.05; ‖ p < 0.01; ¶ p < 0.001; **p-tau values missing for 1 AD patient, 2 DLB patients, and 1 VaD patient

Figure 2. Scatterplots of CSF analytes. CSF concentrations of Aβ42 (A), t-tau (B), p-tau (C), and MHPG (D) in each patient group.
the AD group compared with all other groups \((p < 0.001)\), but no significant differences in p-tau between the DLB, VaD, or FTD groups were observed. Finally, the DLB group had significantly lower MHPG levels compared with the AD group \((p < 0.01)\) and also tended to be lower in the DLB group than in the VaD \((p = 0.057)\) and FTD \((p = 0.099)\) groups, but did not differ significantly between other groups.

In the AD group compared with all other groups \((p < 0.001)\), but no significant differences in p-tau between the DLB, VaD, or FTD groups were observed. Finally, the DLB group had significantly lower MHPG levels compared with the AD group \((p < 0.01)\) and also tended to be lower in the DLB group than in the VaD \((p = 0.057)\) and FTD \((p = 0.099)\) groups, but did not differ significantly between other groups. Among the DLB patients, two subgroups with “high MHPG” and “low MHPG” were unexpectedly observed on the vertical scatter plot (Figure 2). Student’s t-tests did not reveal any significant differences between these 2 groups with respect to \(\text{A}_\beta_{42} \), t-tau, and p-tau, or age, nor did the groups differ in gender distribution (5 males and 2 females in each subgroup). Due to insufficient data we were unable to determine whether these differences correlated with disease duration or cognitive function.

**Diagnostic accuracy of CSF parameters**

ROC analysis (Table 2) showed that the currently used combination of \(\text{A}_\beta_{42} \), t-tau, and p-tau (using model 1 from the previous study: \(y = -5.098 + 0.005 X_{p-tau} + 0.020 X_{t-tau} - 0.002 X_{\text{A}_\beta_{42}}\)) could differentiate between the DLB and AD groups with a sensitivity of 61.9% and specificity of 91.7% \((\text{AUC} = 0.80)\). As anticipated from the results of our previous study \(^{13}\), the addition of MHPG levels (using model 2 of the previous study: \(y = -13.965 + 0.072 X_{p-tau} + 0.021 X_{t-tau} + 0.143 X_{\text{MHPG}} - 0.006 X_{\text{A}_\beta_{42}}\)) to the current combination of \(\text{A}_\beta_{42} \), t-tau, and p-tau resulted in improved diagnostic accuracy \((p < 0.05)\), giving a sensitivity of 65.1% and specificity of 100% \((\text{AUC} = 0.85)\). Additional models created using multivariate logistic regression were tested but our previously tested models afforded the highest diagnostic accuracy. This combination of did not significantly improve the discrimination of DLB from VaD or FTD. Interestingly, the combination used in model 2 also marginally improved the diagnostic value for the differentiation of AD from FTD \((\text{AUC model 1} = 0.73; \text{AUC model 2} = 0.78)\), although sensitivity was slightly reduced from 77% to 73% and specificity rose from 60% to 82.5%. However, comparison of the ROC curves showed that this improvement was not significant \((p = .10)\). To increase the power of the study, we combined our data with finding from our previous study \(^{13}\) and repeated the analyses (refer to Tables S2 and S3 in supplementary data). These results show that MHPG was reduced significantly in DLB compared with AD \((p < 0.001)\), VaD \((p < 0.01)\), and FTD \((p < .01)\), and is consistent with our current findings that the addition of MHPG to the traditional measures of \(\text{A}_\beta_{42} \), t-tau, and p-tau improves the differentiation of DLB from AD, but not FTD or VaD. Although disease duration and MMSE differed significantly between groups in the combined analysis, there were no correlations between MMSE or disease duration and any of the CSF parameters. Furthermore, controlling for age, gender, MMSE, and disease duration did not alter the levels of significance.
Biomarkers distinguish Alzheimer’s disease from dementia with Lewy bodies

Discussion

The current results are in accordance with our earlier study 13 in which we found significantly lower levels of CSF MHPG in DLB compared with AD. In this extended study, we have shown that CSF MHPG levels are also reduced in DLB compared with VaD and bvFTD, and we confirmed that the addition of MHPG to Aβ42, t-tau, and p-tau improves the differentiation of DLB from AD. However, the addition of MHPG to these markers did not improve the differentiation of DLB from either VaD or FTD. Reduced MHPG concentrations in the CSF of DLB patients have been reported previously and are consistent with other studies that identified disturbances of noradrenergic function in patients with DLB 13,34.

Substantial degeneration of locus coeruleus noradrenergic neurons and upregulation of α2-adrenergic receptor (A2R) binding are frequently observed in the brains of both DLB and AD patients but not VaD or FTD patients 16,35. The upregulation of A2R, in particular, is associated with behavioural disturbances and a more extensive upregulation of A2R in DLB may account for the higher prevalence of behavioural problems in DLB compared with AD 35.

Degeneration of the noradrenergic neurons is reflected by reduced levels of norepinephrine in several brain areas of both AD and DLB patients but not in the lumbar CSF of AD patients 14,15. Although CSF norepinephrine is consistently reported as being reduced in DLB compared with controls, unchanged to markedly increased levels have been reported for AD patients 36-38. This may reflect a compensatory mechanism occurring in AD brains that may be influenced by other factors such as insulin or medications 37,38. On the whole, these finding support the notion that reduced CSF MHPG levels reflect more profound disturbances of the noradrenergic system in DLB compared with AD. However, MHPG alone does not adequately discriminate DLB from AD in this study. Interestingly, the DLB patients fell into two distinct group with regard to MHPG levels. The reason for this difference is unclear but was not dependent on age, gender, disease duration, or time between collection and CSF analyses.

Table 2

<table>
<thead>
<tr>
<th>ROC analysis of CSF parameters DLB versus other dementias</th>
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<tbody>
<tr>
<td><strong>CSF variables</strong></td>
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<tr>
<td>-------------------</td>
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<tr>
<td><strong>DLB vs AD</strong></td>
</tr>
<tr>
<td>Model 1</td>
</tr>
<tr>
<td>Model 2</td>
</tr>
<tr>
<td><strong>DL vs VaD</strong></td>
</tr>
<tr>
<td>Model 1</td>
</tr>
<tr>
<td>Model 2</td>
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<tr>
<td><strong>DLB vs FTD</strong></td>
</tr>
<tr>
<td>Model 1</td>
</tr>
<tr>
<td>Model 2</td>
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</tbody>
</table>

*Due to missing data points, not all CSF parameters were available for all patients. †Cut-off refers to the selected value of the individual biomarker or the combination where the two groups can be separated at the indicated sensitivity and specificity.
‡Youden index: sensitivity + specificity - 1.0. §Likelihood ratio: sensitivity / (1 - specificity).
Model 1: \( y = -5.098 + 0.005 X \text{p-tau} + 0.020 X \text{t-tau} - 0.002 X A\beta_{42} \).
Model 2: \( y = -13.965 + 0.072 X \text{p-tau} + 0.021 X \text{t-tau} + 0.143 X \text{MHPG} - 0.006 X A\beta_{42} \).
Measurement of the brain-specific proteins Aβ 42, t-tau, and p-tau have been analysed rigorously for diagnostic accuracy in distinguishing DLB from AD and the CSF levels of Aβ 42, t-tau, and p-tau observed in our study are in agreement with those previously reported in the literature. The reduction of CSF Aβ 42 in DLB patients has been observed previously and was shown to reduce the power of the traditional markers in differentiating AD from DLB. In contrast, a combination of CSF levels of Aβ 42, t-tau, and p-tau shows good diagnostic value for discriminating AD patients from healthy controls and is currently being considered for routine diagnostic use to supplement clinical parameters. Nevertheless, these CSF parameters have been shown to be of limited use in differentiating AD from DLB and other forms of dementia. Indeed, we have again shown that CSF levels of Aβ 42 are significantly reduced in the DLB group and hamper the differentiation of DLB from AD, and the combination of Aβ 42, t-tau, and p-tau provided only moderate discrimination of AD from DLB.

As in our previous study, the addition of MHPG levels improved the diagnostic accuracy compared with traditional markers alone in the discrimination of AD from DLB, albeit to a lesser extent than previously observed. The reduced discriminatory power in this study compared with our previous study may have arise due to the limited number of DLB patients with available CSF data for analysis. Contrary to our hypothesis, the addition of MHPG to Aβ 42, t-tau, and p-tau did not improve the differentiation of DLB from VaD or FTD, even after combining our data set with that of the previous study. Although degeneration of the noradrenergic system has been demonstrated repeatedly in DLB and AD, there has been little or no evidence of degeneration of the locus coeruleus noradrenergic neurons in patients with FTD or VaD. Thus, it was surprising that the addition of MHPG to the traditional markers did not confer any improvement in the discrimination of DLB from these dementias, particularly given that both MHPG and Aβ 42 levels were reduced in DLB compared with VaD and FTD.

However, when we looked more closely at MHPG levels in the FTD subgroups, we found that all but the bvFTD subgroups had MHPG levels indistinguishable from the DLB group. This seems to suggest that noradrenergic neurons may also be affect in FTD but dependent on FTD subtype. This is, however, rather speculative, because the low numbers of patients in each subgroup, particularly the FTD-MND subgroup, limited our ability to generalise these observations. Additional studies in larger cohorts will be needed to further elucidate these observations.

In the AD group, we had a much larger percentage (79%) of female patients than for the other groups (29% - 50%). This may have arisen due to the fact that a greater number of males were included in the previous study (79%), and those patients were excluded from the current analysis, possibly leading to a female selection bias. The larger number of males and younger age for the FTD patients is reflective of greater predominance in males and an earlier age of onset of dementia as established by prior research. Repeated analysis with adjustment for age and gender did not markedly alter our findings.

The majority of the patients in our study were classified as probable or possible and 2 autopsy-confirmed cases of DLB were included in the study. We acknowledge that, because neuropathologic confirmation was not available for most cases, misclassification
may have occurred and that some DLB, VaD, and FTD patient could have had comorbid AD. However, accuracy of the final diagnosis was optimised by extensive clinical and ancillary investigations at baseline and extensive follow-up. Furthermore, the t-tau/ $A\beta_{42}$ ratio was significantly lower in the DLB, VaD, and FTD patients compared with the AD patients, suggesting that these patient groups did not have comorbid AD.

Correct diagnosis is crucial for ensuring the provision of timely and appropriate clinical management. Notably, neuroleptics contributed to worsening of physical and cognitive function and increased mortality in patients with DLB and neuroleptic sensitivity is thus considered to be a major feature of DLB. Despite relatively small numbers of patients with DLB, the results are consistent with the previous findings and emphasise the potential for CSF biomarkers to enhance the differential diagnosis of DLB from AD. Thus, these finding warranted further investigation in larger, independent cohorts with a prospective design.

**Acknowledgements**

The authors thank the technicians in the Department of Laboratory Medicine for performing the CSF analyses. This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project LeARN (grant 02N-101). LeARN is a consortium of Philips, Merck/MSD, Virtual Proteins, BAC, Cyclotron BV, LUMC, VUMC, MUMC, and RUMC.

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**Research in Context**

1. Systematic review: We searched previous publications to identify articles describing noradrenergic changes in different forms of dementia. Several studies showed reduced brain levels of norepinephrine and its metabolite MHPG in DLB compared with other forms of dementia and we hypothesised that this difference in MHPG levels may be reflected in CSF and provide a tool for discriminating DLB from other forms of dementia.

2. Interpretation: Our results demonstrated that CSF levels of MHPG are reduced in DLB compared with AD, VaD and FTD and that the addition of MHPG to traditional biomarkers ($A\beta_{42}$, t-tau, and p-tau) leads to optimal discrimination of DLB from AD.

3. Future directions: Our findings warrant validation in a prospective study with long-term follow up or neuropathological confirmation of the diagnosis. Future research is also required to identify additional biomarkers for the accurate discrimination of DLB from dementia disorders other than AD.
References

Biomarkers distinguish Alzheimer’s disease from dementia with Lewy bodies


### Table S1

**Characteristics of the frontotemporal dementia subtypes**

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>bvFTD</th>
<th>nfFTD-PPA</th>
<th>svFTD-PPA</th>
<th>FTD-MND</th>
<th>FTD + park</th>
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</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>7</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Age, years (SD)*</td>
<td>60.0 (10.6)</td>
<td>63.2 (4.8)</td>
<td>63.8 (10.3)</td>
<td>56.0 (15.7)</td>
<td>61.7 (12.7)</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>6/1</td>
<td>5/4</td>
<td>1/2</td>
<td>1/1</td>
<td>3/0</td>
</tr>
<tr>
<td>MMSE, average (SD)</td>
<td>18.8 (4.0), n=4</td>
<td>18.6 (6.1), n=5</td>
<td>10 (→), n=1</td>
<td>26 (→), n=1</td>
<td>2 (→), n=1</td>
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<tr>
<td>Disease duration, months (SD)</td>
<td>17 (27), n=3</td>
<td>4 (2), n=5</td>
<td>2 (→), n=1</td>
<td>1 (→), n=1</td>
<td>NA</td>
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<tr>
<td>CSF parameters †</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHPG, nmol/L (SD)</td>
<td>56.0 (13.1)</td>
<td>44.7 (5.5)</td>
<td>38.3 (6.1)</td>
<td>34.0 (7.1)</td>
<td>48.3 (13.6)</td>
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<tr>
<td>A(^42)</td>
<td>738 (236)</td>
<td>735.9 (225)</td>
<td>529 (180)</td>
<td>619 (217)</td>
<td>666 (264)</td>
</tr>
<tr>
<td>t-tau, ng/L</td>
<td>401 (301)</td>
<td>436 (237)</td>
<td>795 (606)</td>
<td>279 (78)</td>
<td>326 (190)</td>
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<tr>
<td>p-tau, ng/L</td>
<td>65 (36)</td>
<td>64 (26)</td>
<td>95 (39)</td>
<td>37 (18)</td>
<td>41 (18)</td>
</tr>
<tr>
<td>t-tau/A(^42) ratio</td>
<td>0.56 (0.47)</td>
<td>0.65 (0.42)</td>
<td>1.71 (1.24)</td>
<td>0.46 (0.04)</td>
<td>0.52 (0.31)</td>
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</tbody>
</table>

Abbreviations: bvFTD, behavioural variant frontotemporal dementia; nfFTD-PPA, nonfluent type FTD; PPA, primary progressive aphasia; svFTD-PPA, semantic variant FTD-PPA; FTD-MND, motor neuron disease; MND, motor neuron disease; SD, standard deviation; M, males; F, females; MMSE, Mini-Mental State Examination; NA, not available; CSF, cerebrospinal fluid; MHPG, 3-methoxy-4-hydroxyphenylethyleneglycol.

*At time of lumbar puncture.
†p-values for differences using one-way analysis of variance. Gender distribution was analysed using chi-square test.
‡p<0.05.

### Table S2

**Characteristics of the combined diagnostic groups**

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>DLB</th>
<th>AD</th>
<th>VaD</th>
<th>FTD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>36</td>
<td>109</td>
<td>15</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age, years (SD)*</td>
<td>72.1 (8.7)</td>
<td>72.5 (8.8)</td>
<td>76.5 (4.8)</td>
<td>61.6 (8.4)</td>
<td>&lt;0.001</td>
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<tr>
<td>Gender, M/F</td>
<td>44/14</td>
<td>47/62</td>
<td>10/5</td>
<td>17/9</td>
<td>&lt;0.001</td>
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<tr>
<td>MMSE, average (SD)</td>
<td>23 (4.4), n=28</td>
<td>20 (4.6), n=106</td>
<td>18 (3.7), n=12</td>
<td>18 (7.3), n=13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Disease duration, months (SD)</td>
<td>34 (25), n=27</td>
<td>15 (16), n=61</td>
<td>17 (15), n=12</td>
<td>7.3 (14), n=10</td>
<td>&lt;0.01</td>
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<tr>
<td>CSF parameters †</td>
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</tr>
<tr>
<td>MHPG, nmol/L (SD)</td>
<td>38.3 (13.5)</td>
<td>50.0 (12.8)</td>
<td>49.9 (15.2)</td>
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<td>A(^42)</td>
<td>528 (184)</td>
<td>474 (162)</td>
<td>568 (168)</td>
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<tr>
<td>t-tau, ng/L</td>
<td>246 (128)</td>
<td>706 (440)</td>
<td>380 (269)</td>
<td>443 (303)</td>
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</tr>
<tr>
<td>p-tau, ng/L**</td>
<td>52 (16)</td>
<td>109 (49)</td>
<td>54 (19)</td>
<td>64 (31)</td>
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<tr>
<td>t-tau/A(^42) ratio</td>
<td>0.54 (0.34)</td>
<td>1.669 (1.29)</td>
<td>0.77 (0.80)</td>
<td>0.72 (0.62)</td>
<td></td>
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</tbody>
</table>

Abbreviations: DLB, dementia with Lewy bodies; AD, Alzheimer’s disease; VaD, vascular dementia; FTD, frontotemporal dementia. Refer to Table S1 for other abbreviations.

*At time of lumbar puncture.
†p-values are presented for differences using one-way analysis of variance in the case of Gaussian-distributed data or Kruskal-Wallis in the case of non-Gaussian-distributed data (disease duration only). Gender distribution was analysed using the chi-square test.
‡Analysis of variance of transformed data was performed to test differences in MHPG, t-tau, and p-tau, and the Kruskal-Wallis test was used to test for differences in A\(^42\) for each group compared with DLB.
§p<0.05; ||p<0.01; ¶p<0.001; **p-tau values were missing for 1 AD patient, 2 DLB patients, and 1 VaD patient.
### Table 3

ROC analysis of CSF parameters DLB versus other dementias in combined data

<table>
<thead>
<tr>
<th>CSF variables</th>
<th>Number of patients*</th>
<th>Cut-off † point</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC (95% CI)</th>
<th>Youden index ‡</th>
<th>Likelihood ratio§</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLB vs AD</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>DLB n=31, AD n=107</td>
<td>&gt;2.102</td>
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<tr>
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Abbreviations: AUC, area under the curve. Refer to Table S1 for other abbreviations.

*Due to missing data points, not all CSF parameters were available for all patients.

†Cut-off refers to the selected value of the individual biomarker or the combination where the 2 groups could be separated at the indicated sensitivity and specificity.

‡Youden index: sensitivity + specificity - 1.0.

§Likelihood ratio: sensitivity / (1 - specificity).

|| Model 1: \( y = -5.098 + 0.005 \times \text{p-tau} + 0.020 \times \text{t-tau} - 0.002 \times \text{Aβ}_42 \)

¶Model 2: \( y = -13.965 + 0.072 \times \text{p-tau} + 0.021 \times \text{t-tau} + 0.143 \times \text{MHPG} - 0.006 \times \text{Aβ}_42 \)
Biomarkers distinguish Alzheimer's disease from dementia with Lewy bodies
Chapter 3

Elevated oxidised Pin1 /total Pin1 levels in early stage AD pathology

Megan K Herbert, Marcel M Verbeek, Benno Küsters, H Bea Kuiperij

Manuscript in preparation

"That's the effect of living backwards," the Queen said kindly: "it always makes one a little giddy at first--"
Abstract

Oxidative stress occurs in many neurodegenerative diseases including Alzheimer’s disease (AD) and evidence suggests that specific proteins are oxidised in individual diseases. In AD in particular, the peptidyl prolyl isomerise Pin1, has been shown to be sensitive to oxidative stress. Thus measures of oxidised proteins such as Pin1 in human biological samples could represent potential disease-specific biomarkers. Protein carbonylation is considered to be an important marker of oxidative stress. Based on this protein modification we developed a novel, enzyme-linked sandwich immunoassay for the quantification of oxidised Pin1 (oxPin1) in human brain tissue samples. Our results show an increased ratio of oxPin1 to total Pin1 in the hippocampal tissue of patients with early AD pathology compared with controls. This is consistent with increased oxidative stress in the early stages of AD and suggests that the oxPin1/Pin1 ratio could indicate early stage pathology and warrants further investigation in other biological fluids.
Elevated oxidised Pin1 /total Pin1 levels in early stage AD pathology

Introduction

The brain uses more oxygen than any other organ of the body, making it prone to damage by reactive oxygen species (ROS) produced by its aerobic activity. ROS are produced continuously and play an important role in biological processes such as signal transduction, immune responses and aging (Dickinson and Chang, 2011). An imbalance between endogenous pro-oxidants and antioxidant defences leads to unregulated production of ROS and subsequent oxidative stress (Axelsen et al., 2011) and is implicated in the progression of neurodegenerative brain diseases including Alzheimer’s disease (AD) (Barnham et al., 2004).

AD is characterised by progressive memory loss and cognitive deterioration, and is the most common neurodegenerative disease. Post-mortem histopathological examination of the brain reveals the presence of pathological hallmarks of AD, amyloid-beta (Aβ)- containing senile plaques (SPs) and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein (Braak and Braak, 1991; Mirra et al., 1991). Another common feature of AD brain is extensive oxidative stress, manifesting as lipid peroxidation, DNA hydroxylation and protein carbonylation (Barnham et al., 2004). Moreover, Aβ proteins in the presence of copper are directly capable of producing ROS, particularly H₂O₂, and likely contribute to oxidative stress in AD (Butterfield et al., 2001; Feaga et al., 2011).

There is increasing evidence that specific proteins are oxidised in individual neurodegenerative diseases (Butterfield et al., 2006; Sultana et al., 2010). In AD, proteins most vulnerable to oxidative stress include the peptidyl-prolyl isomerase, Pin1 (Sultana et al., 2006). This is of particular importance to AD, since Pin1 binds to phosphorylated tau (p-tau), amyloid precursor protein (APP) and glycogen synthase kinase-3β (GSK3β), all of which play a significant role in its pathogenesis. Pin1 binds specifically to proteins with phosphorylated serine (Ser) or threonine (Thr) residues preceding a proline residue. Upon binding, Pin1 catalyses the isomerisation of the protein at the proline residue, twisting it from cis to trans conformation (Lopez et al., 2003). Upon Pin1 binding at Thr231, tau is isomerised to trans conformation, facilitating its dephosphorylation by the trans-specific protein phosphatase 2A (PP2A) (Landrieu et al., 2011). Similarly, processing of APP via the non-amyloidogenic pathway is favoured when APP is in trans-conformation and binding of Pin1 to APP at Thr668 reduces production of Aβ₄₂ (Davies et al., 2001). Pin1 is also responsible for inhibition of glycogen-synthase kinase 3β (GSK-3β), which is involved in the phosphorylation of both APP and tau (Ma et al., 2012; Martić et al., 2012). By binding to GSK-3β, Pin1 further reduces phosphorylation of both APP and tau thus further reducing amyloidogenic processing of APP and hyperphosphorylation of tau. When Pin1 is oxidised, its activity is restrained and can no longer bind to its substrates. Hence, the absence of Pin1 may correlate with enhanced amyloidogenic APP processing and increased Aβ₄₂ production together with hyper-phosphorylation of tau and subsequent formation of NFTs (Butterfield et al., 2006; Sultana et al., 2006).

In this study, we aimed to develop a simple, effective means for measuring levels of oxidised Pin1 in human brain tissue and to determine the extent of Pin1 oxidation in AD compared with controls. For this purpose, we developed an ELISA for the measurement of oxidised Pin1 (oxPin1) in brain tissue and measured levels of oxPin1 at different stages of
Alzheimer's disease pathology, compared with cognitively normal controls.

Materials and Methods

Tissue Samples

Frozen human hippocampal tissue samples from controls and patients with AD pathology were obtained from the Pathology department, collected using standard protocols, and informed consent had been obtained according to European guidelines. Additional hippocampal samples were obtained from the Netherlands Brain Bank (NBB, Netherlands Institute for Neuroscience Amsterdam), collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. Tissue samples were taken as quickly as possible after autopsy and rapidly frozen in liquid nitrogen. Post-mortem diagnosis and grading was performed and judged by a neuropathologist, according to the criteria established by Braak & Braak (all samples) and CERAD (for Radboud University Medical Centre patients only) (Braak and Braak, 1991; Braak and Braak, 1995; Mirra et al., 1991). Clinical diagnosis of AD was based on the clinical criteria of probable AD (McKhann et al., 1984; Waldemar et al., 2007). Patient characteristics for all patients are described in Table 1.

Table 1

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<td>Early AD pathology</td>
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<table>
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<tr>
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Values are presented as mean (standard deviation) except where otherwise indicated; 1 p-values obtained using ANOVA
Protein extraction from brain tissue was performed by suspending serial cryosections of hippocampus or cortex (10 µm thickness) in cold 5M guanidine hydrochloride (GuHCl)/50 mM Tris-HCl, pH 8.0 containing a protease inhibitor cocktail (Complete Mini, EDTA free, Merck Millipore, Darmstadt, Germany) and vortexing until the tissue was fully lysed. Samples were centrifuged for 20 minutes (16,000 x g at 4°C) and the supernatant was stored at -80 °C in clean polypropylene tubes. Total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Production and purification of recombinant Pin1 protein

Recombinant Pin1 protein was produced using a construct of Pin1 cDNA cloned in the pET-46 Ek expression vector (“Merck KGaA, Darmstadt, Germany) and OneShot™ BL21 (DE3) Star™ cells (Life Technologies, Carlsbad, CA, USA) by isopropyl β-D -thiogalactoside (ITPG) induction. His-tagged recombinant protein was purified under native conditions using a Nickel Nitrilotriacetic acid (Ni-NTA) chromatography column (Qiagen, Hilden, Germany) and elution with imidazole. The protein was cleared of imidazole using dialysis with phosphate buffered saline and subsequently concentrated using a 9 kDa cut-off protein concentrator according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). The final protein concentration was determined using the BCA protein assay kit.

Oxidation of recombinant Pin1 protein

Purified Pin1 protein (80.8 µL; 0.5 µg/mL; estimated purity ~95% based on SDS-PAGE gel analysis) was incubated with an oxidation solution containing 0.2mM Fe(II)SO4, 10mM H2O2 and 0.3mM EDTA in PBS (pH 7.4) in a final volume of 100 µL for 3h (Sultana et al., 2006). One microliter of 40µM butylated hydroxytoluene in methanol was added to stop the reaction giving a final (theoretical) protein concentration of 0.4 µg/mL. The mixture was dialysed against PBS (pH 7.4) at 4°C using a mini slide-a-lyser cassette (2 kDa cut-off; Thermo Scientific, Rockford, IL, USA) for 2 hours and then overnight in fresh, cold PBS. Following dialysis, the sample was divided into aliquots and stored at -80°C.

SDS-PAGE analysis of Pin1

Recombinant Pin1 protein (1 µg) and an equal volume of oxidised recombinant Pin1 protein were mixed with 4X reducing sample buffer (25% (w/v) glycerol, 2% (w/v) SDS, 62.5 mM Tris–HCl, pH 6.8, 32 mM DTT and 0.005% (w/v) bromophenol blue) and PBS to a final volume of 16 µl. Samples were then heated to 95°C for 5 minutes, loaded on to SDS-PAGE gels with 4% acrylamide stacking gel and 12% acrylamide running gel, and electrophoresed at 200V. Gels were stained with Coomassie brilliant blue (R250, Serva) stain for 45 minutes. The Precision Plus Protein™ Standard (All Blue) molecular weight marker (10-250 kDa; Biorad Laboratories, Inc., California, USA) was used to approximate protein size. Protein bands were scanned using a BioRad Gel Doc 2000 apparatus and density measurements were used to calculate oxPin1 concentration in comparison to non-oxidised protein (Quantity One programme – BioRad v.4.2.1).
Direct ELISA for carbonylated proteins

A direct ELISA for the measurement of carbonyl content in the protein samples with minor modifications from the original protocol (Korolainen et al., 2010) was used to confirm protein carbonylation as a measure of protein oxidation. Protein samples derivatised with 2,4-dinitrophenyl hydrazine (5 µM DNPH; 45 min) in 6M GuHCl, pH 2.4 (1:3 ratio) were absorbed onto an ELISA plate. The hydrazone adducts, 2,4-dinitrophenyl (DNP), were detected using a biotinylated antibody directed against DNP (Anti-Dinitrophenyl-KLH, Rabbit IgG Fraction, Biotin-XX Conjugate; Molecular Probes®, Oregon, USA) and the extent of protein oxidation was assessed by comparison with an oxidized BSA standard. Oxidized and reduced BSA were prepared as previously described (Davies et al., 2001).

OxPin1 sandwich ELISA

OxPin1 levels in brain tissue were quantified using a novel homemade sandwich ELISA with oxidised recombinant Pin1 as standard. ELISA plates (F96 cert. MaxiSorp™ Immunoplate; Nunc, Roskilde, Denmark) were incubated overnight at 4°C with polyclonal rabbit anti-DNP (2 µg/mL in 1M NaHCO₃, pH 9.6; Life Technologies) and washed (5 X 300 μL, 0.05% Tween-20/phosphate buffered saline (PBST)) between all incubation steps. Plates were blocked with 0.1% BSA/PBS, pH 7.4 (250 µL). Samples, standards (oxPin1, stock = 0.2µg/µL) and blank (PBS) were derivatised with 5 µM DNPH in 6M GuHCl, pH 2.4 (1:3 ratio), vortexed, incubated for 45 minutes in the dark, and subsequently diluted in sample diluent (1mM EDTA, 0.5% Triton X-100 in PBS). The highest standard (50 ng/mL) was serially diluted in sample diluent to obtain a 6-point standard curve. Standards, blank (PBS) and tissue samples (100 µL/well) were incubated for 2 hours at room temperature (RT) with agitation (650 rpm). An additional wash step (1:1 Ethanol:PBS) was used to ensure removal of unbound DNPH that could react with the detection antibody. Detection antibody (250 ng/mL biotinylated polyclonal goat anti-Pin1; R&D Pin1 DuoSet, #DYC2294) was applied for 1 hour followed by 20 min incubation with 100 µL of streptavidin-HRP complex (1:200 R&D DuoSet kit). The colour reaction was developed using TMB substrate (30 minutes at RT), stopped with 0.5 M H₂SO₄ (50µl) and read at 450nm (Tecan Sunrise ELISA plate reader and Magellan data analysis software).

Total Pin1 sandwich ELISA

Pin1 levels in brain tissue (diluted 1:25) were measured using a commercially available ELISA kit (R&D DuoSet® IC for total Pin1; Abingdon, UK) according to the manufacturer’s instructions.

Statistical Analysis

Between groups comparison was performed using Kruskal-Wallis tests for non-Gaussian distributed data (oxPin1) and one-way analysis of variance (ANOVA) or Student’s t-test for Gaussian-distributed data. Analysis of covariance (ANCOVA) was performed to control for covariant influences such as age, gender and post-mortem delay.
Results

Confirmation of Pin1 oxidation

Several oxidation conditions were tested and we found that oxidation using 0.2mM Fe(II) SO4, 10mM H2O2, and 0.3mM EDTA gave the optimal results with respect to oxidation level versus protein degradation. Still, SDS-PAGE gel analysis (Figure 1A) suggested that Pin1 was partially degraded after oxidation. Using band density measurements we calculated the concentration of full-length oxidised Pin1 to be 0.2 μg/μL. The oxidised protein migrated more slowly through the gel than non-oxidised protein, indicating an increased size or altered charge likely resulting from addition of carbonyl side chains during the oxidation procedure. Oxidation of Pin1 was confirmed using a direct ELISA for detection of carbonyl side chains. Compared with untreated Pin1, reduced Pin1, reduced BSA, and PBS treated with DNPH, high OD450 values in the direct ELISA obtained for oxPin1 and oxidised BSA confirmed their oxidation status (Figure 1B). Furthermore, the OD450 value for reduced Pin1 did not differ significantly from untreated Pin1 suggesting that untreated Pin1 was not oxidised.

Figure 1. Analysis of Pin1 oxidation. A) SDS-PAGE analysis (visualised with CBB staining) of Pin1 and oxidised Pin1. Recombinant Pin1 runs as a 17kDa product (Lane 1) whereas oxPin1 has a slightly higher molecular weight (Lane 2). B) Direct ELISA for carbonyl proteins. The high OD 450 values obtained from the direct ELISA for carbonyl proteins shows oxidation of oxidised BSA (oxBSA) and oxidised Pin1 (oxPin1) whereas the very low signals for reduced BSA (rBSA), non-oxidised Pin1 (Pin1) and the derivatised PBS blank (PBS) show that these samples contain minimal carbonyl side chains. Bars show average OD values from 4 experiments with error bars showing standard error of the mean.
Validation of the Sandwich ELISA for oxPin1

A typical standard curve for oxPin1 is shown in Figure 2. Linearity was observed up to 50 ng/mL. The lower limit of detection was 2.7 ng/mL. Omission of capture antibody, detection antibody or antigen gave absorbance values resembling the blank confirming the absence of cross-reaction of either capture or detection antibodies with other proteins or each other. It also indicated that neither the biotinylated Pin1 antibody nor brain tissue samples bind non-specifically to the plate.

Figure 2. Standard line – oxPin1 sandwich ELISA. The graph shows average OD values for each oxPin1 standard taken from 7 consecutive experiments. Bars show standard error of the mean for each standard.

Levels of total protein, Pin1 and oxPin1 in brain extracts

Grouped patient demographics and protein levels are reported in Table 1. Patients were divided into groups and analysed according to Braak stage pathology as follows: Controls (Braak 0-2), early AD pathology (Braak 3-4) and advanced AD pathology (Braak 5-6) (Hyman and Trojanowski, 1997). Levels of Pin1 and oxPin1 reported in the main text are reported per µg of total protein. The clinical characteristics of individual patients are provided in supplementary Table S1.
Elevated oxidised Pin1 /total Pin1 levels in early stage AD pathology

Analysis of hippocampal tissue according to the degree of pathology, showed that Pin1 tended to be correlated with post-mortem delay in controls ($r=0.872$, $p=0.054$) and was correlated with age in advanced AD ($r=0.789$, $p=0.012$). No other correlations between protein measurement and age, gender or post-mortem delay were observed. When using ANCOVA to control for both age and post-mortem delay, Pin1 levels were significantly higher in advanced AD pathology compared with both early pathology ($p<0.01$) and controls ($p<0.05$; Figure 3A). The oxPin1:Pin1 ratio was significantly higher in early pathology than controls ($p<0.05$) and advanced AD pathology ($p=0.01$; Figure 3C).

**Figure 3.** Protein levels in hippocampal tissue. Levels of Pin1 per total protein (TP) (A) are increased in advanced AD pathology (Braak stage 5-6) compared with both controls and early AD pathology (Braak stage 3-4) whereas levels of oxidised Pin1 (B) are not different between the groups. The ratio of oxPin1 to Pin1 (C) is increased in early AD pathology compared with controls and advanced AD pathology. Significance levels are those obtained from ANCOVA analysis: * $p<0.05$, ** $p<0.01$.

**Discussion**

Pathologically, Alzheimer’s disease is characterised by the presence of abundant NFTs and amyloid-β containing SPs. Pin1 is a protein that functions in inhibiting the processes leading to the formation of these pathological lesions. Binding of Pin1 to p-tau facilitates its dephosphorylation and, in addition, Pin1 may reduce the phosphorylation of tau by inhibiting GSK-3β, a major tau kinase. Furthermore, binding of Pin1 to APP may reduce its amyloidogenic processing. Thus, reduced function of Pin1 may accelerate the production of Aβ and p-tau, and hence, SPs and NFTs. A possible mechanism of Pin1 dysfunction is its oxidation, as enhanced oxidative stress is another key feature of AD (Axelsen et al., 2011) and Pin1 is a protein that seems to be particularly vulnerable to oxidation, which may lead to its reduced function (Butterfield et al., 2006; Sultana et al., 2006).
We designed a novel sandwich ELISA for the purpose of measuring oxPin1 in biological samples and illustrated its use by assessing levels of oxPin1 in human hippocampal samples from AD patients and healthy controls. We successfully demonstrated that oxPin1 was measurable in human hippocampal samples using the developed ELISA. Our findings substantiate the need for further investigation of the role of Pin1 and oxPin1 in the pathophysiology of AD.

Interestingly, we observed a ~40% increase in Pin1 levels in the hippocampus of patients with advanced AD pathology compared with controls and a ~19% decrease in early stage AD pathology compared with controls. The increase in Pin1 levels in the advanced pathology samples was surprising as we expected from previous research that Pin1 levels would be generally decreased in AD (Lu et al., 1999; Sultana et al., 2006). However, a more recent report using immunohistochemical methods showed redistribution and deposition of Pin1 into neuronal granules in the brains of patients with advanced AD pathology compared with controls (Dakson et al., 2011). Rather than a reduction of Pin1, per se, in these tissues the immunohistochemical investigations tended to suggest an abnormal accumulation of Pin1 that might support our finding of increased Pin1 in advanced AD pathology (Dakson et al., 2011). Since Pin1 is known to be localised in the nucleus we used a GuHCl protocol to extract proteins from the brain tissue. This procedure should enable extraction from all cell fractions including any Pin1 that might have been accumulated abnormally in granules. The previous study also found some correlation, although weak, between severity of Pin1 granules and NFTs in patients with Braak stages 5-6 (Dakson et al., 2011) hinting that levels of Pin1 may be associated with pathological changes. Therefore, it may be interesting in future studies to investigate, using combined techniques, whether oxPin1 might be associated with Pin1 granules or NFTs. Finally, observation of increased levels of Pin1 in advanced stage AD may be a response of the brain to counteract the pathological accumulation of p-tau and Aβ proteins.

In the current study, differences in oxPin1 and Pin1 levels between the groups hinted that the ratio of oxPin1:Pin1 might be more informative than individual measurement of Pin1 or oxPin1 alone. Indeed, when we analysed the ratio of oxPin1:Pin1 in human hippocampal samples we observed an increased ratio in the hippocampi of patients with early AD pathology (Braak stage 3-4) compared with controls (Braak stage 0-2; p = 0.032). This is consistent with the notion that Pin1 is not only oxidised in AD, but that this occurs early in the disease process. Somewhat unexpectedly, we did not observe an increase in the ratio of oxPin1:Pin1 in the patients with advanced AD pathology compared with controls which contradicts previous reports (Butterfield et al., 2006; Sultana et al., 2006). However, there are fundamental differences between the studies in that we classified our samples according to Braak staging as opposed to using clinical diagnoses as in these previous studies and this may provide an explanation for the differences observed between studies.

We argue that classifying patients according to Braak stage was most appropriate for the current study since we were primarily interested in investigating the relationship between levels of oxPin1 and the pathological features of AD as defined by Braak staging. In support of this argument our finding that oxPin1:Pin1 is increased in the hippocampus of early AD pathology confirms suggestions that changes arising from oxidative stress
occur early in the pathophysiology of AD (Butterfield et al., 2006; Sultana et al., 2010). This is relevant in consideration of other research showing the involvement of Pin1 in both the direct regulation of tau phosphorylation and the indirect regulation via GSK3β and PP2A. Together, observations that Pin1 is oxidised early in AD, and likely leads to its dysfunction, helps to confirm that oxidation of Pin1 may lead to enhanced formation of NFTs as it can no longer bind to and dephosphorylate tau.

We acknowledge that relatively small numbers of hippocampal tissues limit our ability to generalise the findings, however, we believe that the main purpose of demonstrating the use of our developed ELISA for the measurement of oxPin1 in human hippocampal samples has been adequately achieved. What’s more, the design of our ELISA using an anti-DNP antibody to capture all carbonylated proteins and specifically detect a target protein using a protein-specific detection antibody enables its adaptation for measurement of other potential disease-specific oxidised proteins such as glutamine synthetase in AD (Butterfield et al., 2006), and DJ-1 and alpha-synuclein in Parkinson’s disease (Andersen, 2004; Choi et al., 2006; Glaser et al., 2005). Such studies may also reveal if protein oxidation is a global process in early stage AD pathology not restricted to Pin1.

In conclusion, we have developed a novel sandwich ELISA for the measurement of oxPin1 in human brain tissue and shown that the ratio of oxPin1:Pin1 is increased in the hippocampus of early stage Alzheimer pathology compared with both controls and advanced AD pathology. Further development of the assay design will enable multifunctional use for the quantification of oxidised proteins in tissues and biological fluids that may be used in investigating the role of oxidised proteins in a range of neurodegenerative diseases, particularly in which disease-specific protein oxidation has been implicated.

Acknowledgements

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Disclosures

The authors declare no conflicts of interest.
References


## Supplementary information

### Supplementary Table S1. Individual patient characteristics

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Elevated oxidised Pin1 /total Pin1 levels in early stage AD pathology
Photo series - Part 3: Patients with Parkinson's disease often have difficulty initiating movement. These photos are intended to represent the delay in initiation of movement for simple tasks such as answering the telephone.
Part 3

The use of CSF biomarkers for the differential diagnosis of movement disorders

"The hurrier I go, the behinder I get"
"Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"
Abstract

Objective: The differentiation between multiple system atrophy (MSA) and Parkinson's disease (PD) is difficult, particularly in early disease stages. Based on previous studies, we aimed to evaluate the diagnostic value of neurofilament light chain (NFL), fms-like tyrosine kinase ligand (FLT3L) and total tau protein (t-tau) in cerebrospinal fluid (CSF) as biomarkers to discriminate MSA from PD.

Methods: Using commercially available enzyme-linked immunoassays (ELISAs), we measured CSF levels of NFL, FLT3L and t-tau in a discovery cohort of 36 PD patients, 27 MSA patients and 57 non-neurological controls and in a validation cohort of 32 PD patients, 25 MSA patients, 15 progressive supranuclear palsy (PSP) patients, 5 patients with corticobasal syndrome (CBS), and 56 non-neurological controls. Cut-offs obtained from individual assays and binary logistic regression models developed from combinations of biomarkers were assessed.

Findings: CSF levels of NFL were substantially increased in MSA and discriminated between MSA and PD with a sensitivity of 74% and specificity of 92% (AUC = 0.85) in the discovery cohort and with 80% sensitivity and 97% specificity (AUC = 0.94) in the validation cohort. FLT3L levels in CSF were significantly lower in both PD and MSA compared to controls in the discovery cohort, but not the validation cohort. T-tau levels were significantly higher in MSA than PD and controls. Addition of either FLT3L or t-tau to NFL did not improve discrimination of PD from MSA above NFL alone.

Interpretation: Our findings show that increased levels of NFL in CSF offer clinically relevant, high accuracy discrimination between PD and MSA.
Introduction
Parkinson’s disease (PD) is the most common movement disorder with a typical age of onset around 60 years although some patients (~3-5%) develop PD before the age of 40\(^1\). PD is characterised by four cardinal motor features: involuntary tremor, postural instability, bradykinesia and rigidity\(^2\). Non-motor features such as cognitive disturbances, depression, mild autonomic dysfunction (including orthostatic hypotension) and disordered sleep commonly accompany these motor symptoms\(^3\).

Multiple system atrophy (MSA) is a relatively rare and sporadic adult-onset disease characterised by a variable combination of parkinsonism, cerebellar ataxia, autonomic dysfunction (particularly orthostatic hypotension), and pyramidal signs\(^4\). MSA is commonly misdiagnosed as PD, particularly in early disease stages, because of overlapping symptoms, occasionally good responsiveness to dopaminergic treatment in MSA, and similar age of onset, typically around 60 years\(^1,4\). However, MSA progresses more rapidly than PD and is associated with a much poorer quality of life\(^5\). Moreover, the response to levodopa, although variable, is generally poor and may lead to worsening of orthostatic hypotension in some MSA patients\(^6\). A reliable biomarker capable of clearly distinguishing between MSA and PD would have great clinical and diagnostic value.

In a recent study, the fms-like tyrosine kinase ligand (FLT3L), was identified as a potential cerebrospinal fluid (CSF) biomarker to differentiate MSA from PD with high accuracy\(^7\). In addition, CSF levels of neurofilament light chain (NFL) and total tau protein (t-tau) have been shown to discriminate MSA from PD\(^8,9\). However, these findings require confirmation. In the current study, we aimed to determine which of these CSF biomarkers (NFL, t-tau or FLT3L), or combination of biomarkers, could provide optimal discrimination of MSA from PD.

Materials and Methods
Patients
The present study was performed at the Radboud University Medical Centre (Nijmegen, the Netherlands). We studied patients initially referred to our tertiary movement disorder centre between December 2000 and November 2008 (Figure 1), with a hypokinetic rigid syndrome of uncertain diagnosis at presentation, and who received a subsequent diagnosis of PD or MSA. Initial clinical diagnosis was established by a neurologist specialised in movement disorders according to current diagnostic criteria for PD\(^10\) and MSA\(^11\) and underwent extensive neurological examination, including patients who had been enrolled in a previous study in which they were studied prospectively for three years (57% of MSA and 86.8% of PD patients). For these patients, diagnosis was established by two neurologists specialised in movement disorders and patients underwent extensive neurological examination together with imaging studies at initial visit and again after three years (Supplementary Methods). Ten MSA patients and 5 PD patients have been described earlier\(^9,12\) but the CSF parameters reported in the current study were not previously reported. The remaining 8 MSA and 4 PD patients were incidental cases for whom case review follow-up was performed by a neurologist (author MBA).
Disease severity was established using the (modified) Hoehn and Yahr (H&Y)\textsuperscript{13} stages and Unified Parkinson’s Disease Rating Scale (UPDRS)\textsuperscript{14}; ataxia severity was assessed using the International Cooperative Ataxia Rating Scale (ICARS)\textsuperscript{15}. Final diagnosis was confirmed by case review of up to nine years after initial visit. Controls consisted of patients who had been referred to our Neurology Department during the period 2001 to 2009, who underwent lumbar puncture as part of the diagnostic process, and who had been confirmed as having no neurological disease.

For the discovery group we analysed CSF samples of PD and MSA patients obtained between 2001 and 2004, and controls consisted of patients confirmed as having no neurological disorder and with lumbar punctures obtained between 2001 and 2006. To validate our findings, we examined CSF in additional MSA and PD patients with lumbar punctures obtained between 2005 and 2008, and control CSF obtained between 2007 and 2010. An additional group of patients with progressive supranuclear palsy (PSP, n= 15) and corticobasal syndrome (CBS, n= 5) with previously unreported, retrospective CSF NFL levels were included to show differences in NFL levels between PD patients

**Figure 1.** Flowchart of patient inclusion in this study. PD: Parkinson’s disease, MSA: multiple system atrophy, PSP: progressive supranuclear palsy, CBD: corticobasal degeneration, CSF: cerebrospinal fluid, n: number. CSF was obtained during the initial diagnostic assessment upon presentation.
and other atypical parkinsonisms (Supplementary Table 5 and Figure 1). Initial clinical
diagnosis for these patients was established by a movement disorders specialist using
current diagnostic criteria for PSP and CBS. Lumbar puncture samples from all MSA
and PD were analysed for all CSF parameters to determine and validate the utility of these
parameters in discriminating PD from MSA. Controls in the discovery group were tested
for all CSF parameters for comparison with PD and MSA patients. We also measured NFL
and FLT3L levels in a second group of controls in the validation cohort to obtain additional
reference values for these parameters.

CSF samples and analysis
CSF samples obtained by lumbar puncture were collected in polypropylene tubes,
centrifuged (5 minutes, 860g at room temperature), and stored at -80°C. Patient information
was decoded to maintain confidentiality. Undiluted CSF samples were measured in
duplicate using commercially available enzyme-linked immunosorbent assays (ELISAs)
for Human FLT3L (R&D Systems, Abingdon, UK), NFL (NF-light® Neurofilament ELISA RUO;
a gift from UmanDiagnostics, Sweden) and t-tau (INNOTEST hTau, Innogenetics N.V.,
Ghent, Belgium). ELISAs were performed according to manufacturer’s instructions except
the capture antibody for the FLT3L ELISA was used at 1 µg/mL.

Statistical Analysis
CSF parameters with non-Gaussian distribution were log transformed and between-group
differences were tested using one-way analysis of variance (ANOVA) followed by Tukey’s
post-hoc test. Mann Whitney U tests were used to compare data with a non-Gaussian
distribution (NFL levels in the discovery group). Spearman rank correlation was used to
determine correlations. We performed analysis of covariance (ANCOVA) to control for
possible confounding variables (e.g. age, gender, disease duration, and disease severity).
Binary logistic regression was used to identify variables contributing to discrimination of
MSA from PD and receiver operator curves (ROCs) were used to determine the diagnostic
accuracy of CSF parameters and models developed from the binary logistic regression.
Statistical analyses were performed using GraphPad PRISM 5 software (San Diego,
California) and SPSS software version 20.0 (Chicago, IL). Comparison of the ROC curves
was performed using MedCalc® software version 12.5.0.0. Bootstrapping analyses using
data from both cohorts was also performed for additional validation of the measures
using Medcalc 12.7.0 9 Trial version.

Results
We had access to 233 CSF samples from 52 MSA patients, 68 PD patients and 113 non-
neurological controls. Of these, 61% (32/52) of the MSA and 87% (59/68) of the PD
patients had been studied prospectively for three years. The discovery group consisted of
36 PD, 27 MSA and 57 controls. Patient characteristics and CSF parameters are reported in
Table 1. Lumbar puncture samples from controls were used to obtain reference values for
NFL, FLT3L and t-tau. In order to confirm the use of NFL, FLT3L and t-tau in discriminating
between PD and MSA we included a validation group consisting of 32 PD and 25 MSA
patients. Since CSF measures of NFL and FLT3L are rather novel, we included 56 additional
controls to obtain additional reference values.
NFL, t-tau and FLT3L levels in CSF of the discovery cohort

In the discovery group, CSF FLT3L levels were significantly lower in PD (38.4 +/- 11.9 ng/L; p<0.01) and MSA (39.3 +/- 12.4 ng/L; p<0.05) compared with controls (47.8 +/-14.3 ng/L) but similar in MSA and PD (Figure 2A). We found significantly higher levels of CSF NFL in MSA (4548 +/- 3206 ng/L) compared with both PD (1350 +/- 915 ng/L, p <0.001) and controls (1503 +/- 619 ng/L, p <0.001) but no differences between PD and controls (Figure 2B). CSF t-tau levels were significantly higher for MSA (335 +/- 164 ng/L) than for PD (242 +/- 190 ng/L; p<0.05; Figure 2C) and compared with our reference values for t-tau in healthy controls 46% of MSA patients and 11.4% of PD patients had elevated (≥350 ng/L) t-tau levels.

We observed that FLT3L was correlated with both NFL and t-tau for the PD patients and controls but not in the MSA group. There was a moderate correlation between NFL and t-tau in PD (r=0.39, p<0.05) but not MSA (r=0.34, p=0.11) or controls (r=0.43, p=0.08). Details of the correlation data are provided in Supplementary Table 1. Four of the PD patients exhibited levels of t-tau that were markedly different from the remainder of the group (Figure 2C) but this was not correlated with MMSE since individual MMSE scores were 30 for 1 patient, 29 for 2 patients and 26 for 1 patient. Despite long-term clinical follow-up (3 to 8.8 years), we can neither rule out, nor confirm, subclinical tauopathy in these patients.

NFL alone provided high discrimination (AUC 0.85) between MSA and PD patients with 74.1% sensitivity and 91.7% specificity. The combination of t-tau and NFL developed in our previous study9 (Model 1: y = NFL+0.15* t-tau; AUC = 0.89) yielded similar sensitivity (75.0%) and specificity (91.2%; AUC = 0.90) for discriminating MSA from PD whereas the combination of FLT3L and NFL (Model 2: y = -1.646+0.001*NFL-0.0308*FLT3L) yielded a sensitivity of 81.8% and specificity of 94.8% (AUC = 0.89). The combination of NFL, FLT3L and t-tau (Model 3: y = -3.054 – 0.001*NFL + 0.003*t-tau – 0.028*FLT3L) yielded a higher AUC (0.92) with increased sensitivity (94.7%) but reduced specificity (83.3%). Comparison of the ROC analyses showed that this improvement was not significantly better at discriminating between MSA from PD than NFL alone (p>0.05).

Gender was not correlated with any CSF parameter for any group. Age was correlated with, or tended to be correlated with, all CSF parameters in both PD and controls but
was not correlated with any CSF parameter in MSA (Supplementary Table 2). Disease duration was not correlated with any CSF parameter for either PD or MSA in the discovery cohort. ICARS and H&Y scores were not correlated with any CSF parameter in either of the patient groups. UPDRS was not correlated with any CSF parameter in the PD group but, intriguingly, showed a significant negative correlation with NFL \( (r=-0.57, p<0.05) \) in MSA. Details of these correlations are provided in Supplementary Table 3. When we repeated our analyses controlling for these factors (age, gender, UPDRS, and disease duration) using ANCOVA, significance levels for NFL were maintained but significance levels for FLT3L and t-tau were not, suggesting that NFL levels are robust but that FLT3L and t-tau levels may be influenced by other factors giving rise to heterogeneity in values.

**Validation of the diagnostic markers**

In the validation cohort we confirmed that CSF levels of NFL were higher in MSA \( (5938 \pm 4267 \text{ ng/L}) \) compared with both PD \( (1103 \pm 442 \text{ ng/L}; p<0.001) \) and controls \( (1290 \pm 664 \text{ ng/L}; p<0.001; \text{ Supplementary Table 4}) \). CSF NFL levels were also significantly higher in other atypical parkinsonisms (AP; 15 PSP and 5 CBS) than in both PD and controls (Supplementary Table 5). This significance was maintained after controlling for age, gender and disease duration with ROC curves ≥ 0.9 (Supplementary Figure 1). FLT3L levels were non-significantly lower in both PD and MSA compared with the controls although a small significant difference between MSA and controls was found after controlling for age, gender, disease duration and disease severity \( (p<0.05) \). As with the discovery group, we also observed higher levels of t-tau in MSA than in PD but this failed to reach significance \( (p=0.06) \). We noted that t-tau levels in both PD and MSA in the validation groups were overall lower than in the discovery group and for MSA the difference t-tau levels in discovery \( (335 \pm 164 \text{ ng/L}) \) versus validation \( (244 \pm 93 \text{ ng/L}) \) was significant \( (p<0.05) \). The methodology used to measure t-tau (Innotest ELISAs) was the same for all patients but CSF samples collected prior to 2004 were analysed retrospectively, which may have influenced our results.

Disease duration was significantly shorter in PD (25.1 months; range 6-84) than MSA (39.0 months; range 12-106) in the validation group, but controlling for this variable using ANCOVA in the analysis of CSF parameters did not alter significance levels.

The models developed using the CSF parameters from the discovery group were applied to the validation group and diagnostic values were calculated using cut-offs obtained from the discovery group. We were able to correctly identify the majority of MSA patients (sensitivity = 80% and specificity = 97%) using NFL alone (AUC = 0.94). Again, ROC curve comparison showed that none of the models significantly improved the discrimination of MSA from PD.

Using bootstrapping analysis of the combined data to further validate our results, we produced an ROC curve for NFL (PD vs. MSA) that was highly comparable with ROC curves from the individual cohorts \( (\text{AUC} = 0.90; \text{ sensitivity} = 77\%; \text{ specificity} = 96\%, \text{ cut-off } >2174 \text{ng/L}) \). Bootstrapping of the combined FLT3L data revealed significantly lower levels of FLT3L in both the PD and MSA groups compared with controls as was observed only in the discovery group but not in the validation group. We found no significant differences in CSF FLT3L levels between PD and MSA patients in the individual cohorts nor when using bootstrapping of the combined data.
Discussion

In the current study, we showed that CSF levels of NFL can be used for clinically relevant discrimination of MSA from PD. These results confirm our previous findings using a different method of detection for NFL⁹ and the findings of a more recent large study using the same ELISA method⁸. Higher t-tau levels for MSA patients in this study confirm similar observation in other studies⁸,⁹,¹² but the contribution of t-tau to the overall discrimination of PD from MSA was not significant. We noted high t-tau values in around 11% of our PD patients and 46% of our MSA patients in the discovery group. However, very few patients in the validation group had high t-tau levels (3% of PD and 8% of MSA). Since the diagnostic value of our previously developed model combining NFL and t-tau (model 1), did not differ between the discovery and validation groups, this variation probably did not adversely influence our results.

We found significantly decreased CSF FLT3L levels in both PD and MSA compared to controls in our discovery cohort, but were unable to confirm these results in the validation cohort. Bootstrapping of the combined data was consistent with the results of the discovery group, revealing significantly lower levels of FLT3L in both PD and MSA compared with controls but showing no significant differences in CSF FLT3L levels between PD and MSA. This contradicts an earlier study showing high accuracy discrimination between PD and MSA using FLT3L alone⁷. As with the previous study, levels of CSF FLT3L were significantly lower in MSA than controls, but we did not observe higher levels of FLT3L in PD. Unlike the earlier study, we did not attempt to exclude patients with possible familial PD so variance could be partly attributable to the inclusion of younger PD patients (<50 yrs in 15/52 PD patients) since we observed a strong correlation between age and FLT3L levels in both PD and controls. After subdivision of PD and MSA for age (i.e. >50 and <50 years), differences between PD and MSA were maintained and we found no differences between young vs. old PD or MSA patients (data not shown).

Neurofilament proteins are essential for maintaining the neuronal cytoskeleton and increased levels of NFL in the CSF of MSA patients likely reflects extensive axonal degeneration⁹. In keeping with earlier findings⁸,⁹, CSF NFL was increased in MSA and aided discrimination of MSA from PD⁹ and, in the current study, NFL alone provided the best tool for discriminating between MSA and PD. The addition of FLT3L and t-tau to NFL analysis improved this discrimination only slightly. However, we observed strong correlations between NFL and FLT3L in PD and controls in both the discovery and validation phases that warrant further investigation to determine the potential function of FLT3L in the central nervous system. The lack of correlation between NFL and FLT3L in the MSA patients suggests that increased levels of NFL were not dependent on changes in FLT3L or vice versa and does not support a role for FLT3L in the pathology of MSA.

FLT3L is a haematopoietic growth factor expressed in various tissues including the brain¹⁸ and has an important role in haematopoietic stem cell survival and proliferation¹⁹. Although FLT3L has a neurotrophic function contributing to increased survival of a subset of post-mitotic neurons¹⁸, its role in neurodegenerative diseases is unknown. In amyotrophic lateral sclerosis (ALS), CSF levels of FLT3L are elevated compared with healthy controls²⁰.
Nerve growth factor (NGF), which normally synergises with FLT3L to exert its neurotrophic effect, also increases the expression of NFL. Since both NGF and NFL are increased in ALS \(^ {21, 22}\), NGF may contribute to elevated levels of FLT3L and NFL as observed in ALS \(^ {20, 23, 24}\). In PD, levels of NGF are reduced \(^ {23}\) and possibly contribute to observed reductions in CSF FLT3L and NFL levels in some patients. However, contrary to previous observations, CSF FLT3L levels alone do not serve as a biomarker for differentiation of MSA from PD.

A major strength of our study is that diagnosis was made prospectively for the majority of PD and MSA patients using detailed neurological examination in combination with imaging studies, and final diagnosis was confirmed after long follow-up by case review. Our findings emphasise a consistency with other studies \(^ {8, 9, 25, 26}\) showing that CSF NFL levels could be a useful adjunct to clinical diagnosis for distinguishing PD from MSA and other atypical parkinsonisms. Since both ours and previous studies have shown significantly increased CSF NFL levels in atypical parkinsonism disorders other than MSA, including PSP and CBS \(^ {8, 25, 26}\), CSF NFL levels do not represent a specific marker for MSA but rather, may be more generally useful for distinguishing PD from atypical parkinsonisms \(^ {8, 9}\). Our results will require confirmation in larger cohorts in future research, with (eventual) pathological confirmation of disease. Further, additional studies will also be required to determine whether NFL levels are influenced by other extraneous influences such as other non-neurological diseases (e.g. cancer) \(^ {27, 28}\) or familial versus sporadic forms of PD, and to determine whether increased NFL levels will be useful for differentiating PD from other APs at early stages of disease.

**Acknowledgements**

Megan Herbert and Marcel Verbeek had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Marcel Verbeek, Bas Bloem and Rianne Esselink were responsible for study concept and design, and study supervision. Megan Herbert was responsible for data and statistical analyses and drafting of the manuscript. Bea Kuiperij was responsible for study supervision. Marjolein Aerts and Marijke Beenes were responsible for data acquisition. All authors critically reviewed and approved the manuscript for submission.

We also thank Alexandra Versleijen and other technicians of the Department of Laboratory Medicine for performing the CSF analyses.

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References


# Supplementary Data

**Supplementary Table 1.** Patient demographic and baseline characteristics – discovery cohort.

<table>
<thead>
<tr>
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<th>PD (n= 36)</th>
<th>MSA (n=27)</th>
<th>Controls (n=57)</th>
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<td>62.6 (9.0)</td>
<td>57.0 (11.5)</td>
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<td>22 (61.1)</td>
<td>15 (55.6)</td>
<td>57.0 (11.5)</td>
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<td><strong>Years of follow-up</strong></td>
<td>5.5 (0-9.2)</td>
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<td>1350 (915)</td>
<td>4548 (3206)</td>
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<td><strong>FLT3L (ng/L)</strong></td>
<td>38.4 (11.9)</td>
<td>39.3 (12.4)</td>
<td>1503 (619)</td>
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<td>242 (190)</td>
<td>335 (164)</td>
<td>47.8 (14.3)</td>
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<tr>
<td><strong>months (range)</strong></td>
<td>43.8 (6 - 158)</td>
<td>43.7 (8 – 96)</td>
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<td><strong>H&amp;Y</strong></td>
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<td>2.7 (1.2); n=14</td>
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<td>32.5 (16.7); n=14</td>
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<td>12.1 (9.5); n=9</td>
<td>p = 0.020</td>
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<td><strong>Cognitive function</strong></td>
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<td><strong>MMSE; mean (SD)</strong></td>
<td>28.5 (1.6)</td>
<td>27.5 (3.5)</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>

SD: standard deviation; H&Y: Hoehn and Yahr score; ICARS: International Cooperative Ataxia Rating Scale; UPDRS: Unified Parkinson’s Disease Rating Scale; N/A: not applicable

* Student’s t-test p-values for PD versus MSA; † At time of lumbar puncture; ‡ At time of inclusion
### Table 2. Comparison of models for discriminating MSA from PD

<table>
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<tr>
<th>CSF variables</th>
<th>Number of patients(^a)</th>
<th>Cut-off (^b) point</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC</th>
<th>Youden index(^c)</th>
<th>Likelihood ratio(^d)</th>
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<tr>
<td>NFL</td>
<td>PD=36, MSA=27</td>
<td>&gt;2315</td>
<td>74.1</td>
<td>91.7</td>
<td>0.854</td>
<td>65.7</td>
<td>8.9</td>
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<td>Model 1(^e)</td>
<td>PD=34, MSA=20</td>
<td>&gt;2388</td>
<td>75.0</td>
<td>91.2</td>
<td>0.879</td>
<td>70.6</td>
<td>8.5</td>
</tr>
<tr>
<td>Model 2(^f)</td>
<td>PD=31, MSA=22</td>
<td>&gt;-0.925</td>
<td>81.8</td>
<td>94.8</td>
<td>0.887</td>
<td>68.9</td>
<td>6.3</td>
</tr>
<tr>
<td>Model 3(^g)</td>
<td>PD=29, MSA=15</td>
<td>&gt;-1.604</td>
<td>94.7</td>
<td>83.3</td>
<td>0.921</td>
<td>78.1</td>
<td>5.7</td>
</tr>
<tr>
<td><strong>Validation</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NFL</td>
<td>PD=32, MSA=25</td>
<td>&gt;2315</td>
<td>80.0</td>
<td>96.9</td>
<td>0.938</td>
<td>76.9</td>
<td>25.6</td>
</tr>
<tr>
<td>Model 1(^e)</td>
<td>PD=32, MSA=24</td>
<td>&gt;2388</td>
<td>76.1</td>
<td>96.9</td>
<td>0.932</td>
<td>76.1</td>
<td>25.3</td>
</tr>
<tr>
<td>Model 2(^f)</td>
<td>PD=32, MSA=23</td>
<td>&gt;-0.925</td>
<td>82.6</td>
<td>96.9</td>
<td>0.969</td>
<td>79.5</td>
<td>26.4</td>
</tr>
<tr>
<td>Model 3(^g)</td>
<td>PD=32, MSA=22</td>
<td>&gt;-1.604</td>
<td>81.8</td>
<td>96.9</td>
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<td>26.2</td>
</tr>
</tbody>
</table>

\(^a\) Due to missing data points, not all CSF parameters were available in all patients; \(^b\) Cut-off refers to the selected value of the individual biomarker or the combination where the 2 groups can be separated at the indicated sensitivity and specificity; \(^c\) Youden index: sensitivity + specificity – 100. \(^d\) Likelihood ratio: sensitivity/(1-specificity)

\(^e\) Model 1: \(y = NFL + 0.15\times t\text{-tau}\)

\(^f\) Model 2: \(y = -1.646 + 0.001\times NFL - 0.03\times FLT3L\)

\(^g\) Model 3: \(y = -3.054 - 0.001\times NFL + 0.003\times t\text{-tau} - 0.028\times FLT3L\)
Supplementary Methods

Prospective, longitudinal analysis of patients

During the period September 2003 until November 2006, consecutive new patients with a hypokinetic rigid syndrome who had been referred to the movement disorders clinic of the Department of Neurology at the Radboud University Medical Centre in Nijmegen, the Netherlands were recruited for a three year prospective study. Inclusion and exclusion criteria are provided in Table M1. Patients diagnosed with Parkinson’s disease (PD) and multiple system atrophy (MSA) were identified for inclusion in the study.

Table M1. Inclusion and exclusion criteria

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Informed consent was obtained and, within 6 weeks of the initial visit, all patients underwent a structured interview, detailed and standardized neurological examination, magnetic resonance imaging (MRI) scan, lumbar puncture, IBZM-SPECT and electromyography (EMG) of the anal sphincter.

Ethics

Medical ethics approval was obtained from the local Institutional Review Board (2002). All patients signed informed consent forms after detailed explanation of the procedures.

Interview and neurological examination

Interview and neurological examination were performed by two independent physicians, not directly involved in patient care. Using a structured interview the following items were assessed: medical history, current medications, presenting complaints and disease progression, most affected body site, balance and fear of falling, activities in daily living, and quality of sleep. In addition, the following clinimetric scales were scored: Unified Parkinson’s Disease rating scale (UPDRS) III and IV and Hoehn and Yahr score,1 International Cooperative Ataxia Rating Scale (ICARS),2 Mini mental state examination (MMSE)3 and frontal assessment battery (FAB) 4 for cognitive assessment, and Composite Autonomic Symptom Scale (COMPASS)5 for autonomic dysfunction.

Follow up

Three years after the inclusion visit, patients were seen in the outpatient clinic for a repeated structured interview and neurological examination by an independent physician. These neurologists were blinded for the results of all ancillary investigations, except MRI which is now nearly routinely used in clinical practice and to the clinical notes of the treating
Neurofilament light chain discriminates multiple system atrophy from Parkinson's disease

A three year follow-up was chosen as previously published data show a very high concordance between neuropathological diagnosis and clinical diagnosis after at least 2 years follow up by a movement disorder specialist (PPV 99%) 6.

Clinical diagnosis

The clinical diagnosis was established in a systematic fashion by two movement disorder specialists, blinded for test results. Patient information was decoded to maintain patient confidentiality and sequentially presented to the panel in the following order: 1) clinical data and clinimetrics (UPDRS, MMSE, FAB, ICARS) upon inclusion, 2) disclosure of MRI results, 3) description of the reaction to dopaminergic medication and 4) disclosure of the clinical data and clinimetrics (UPDRS, MMSE, FAB, ICARS) after 3 years follow-up. Each time the panel—a consensus—established a diagnosis (either PD or AP (not otherwise specified) and the corresponding degree of uncertainty (on a 0-100% rating scale), followed by a more specific diagnosis (e.g. PD, MSA or PSP) always according to the international clinical criteria. (UK Parkinson’s Disease Society Brain Bank clinical diagnostic criteria for PD,7 NINDS-SPSP criteria for PSP, 8 Boeve criteria for CBS9 McKeith Criteria for DLB 10 Gilman criteria for MSA 11 and Zijlmans criteria for VaP12. For the purposes of the current study, final diagnosis was confirmed by case review at extended (up to 9 years) follow-up.

Supplementary references

Chapter 5

CSF levels of DJ-1 and tau distinguish MSA patients from PD

Megan K Herbert, Jorine M Eeftens, Marjolein B Aerts, Rianne AJ Esselink, Bastiaan R Bloem, H Bea Kuiperij, Marcel M Verbeek


"What does it matter where my body happens to be? My mind goes on working all the same. In fact, the more head downwards I am, the more I keep inventing new things"
Abstract
Differential diagnosis between Parkinson’s disease (PD) and multiple system atrophy (MSA) is difficult, particularly at early disease stages, but is important for therapeutic management. The protein DJ-1 is implicated in the pathology of PD but little is known about its involvement in MSA. We aimed to determine the diagnostic value of CSF DJ-1 and tau proteins for discriminating PD and MSA. DJ-1 and total tau levels were quantified in the CSF of 43 PD patients, 23 MSA patients and 30 non-neurological controls matched for age and gender. Patients were part of a study with a 3-year prospective design with extended case-review follow-up of up to 9 years, ensuring maximum accuracy of the clinical diagnosis. Our results showed that CSF DJ-1 levels could distinguish MSA from PD with 78% sensitivity and 78% specificity (AUC = 0.84). The combination of DJ-1 and tau proteins significantly improved this discrimination to 82% and 81% specificity to identify MSA from PD (AUC = 0.92). Our results highlight the potential benefits of a combination of DJ-1 and total tau as biomarkers for differential diagnosis of MSA and PD.
DJ-1 discriminates multiple system atrophy from Parkinson's disease

Introduction
Clinical differentiation of multiple system atrophy (MSA) from Parkinson's disease (PD) is important because progression of MSA is faster and prognosis is poorer than for PD. However, differential diagnosis is difficult due to overlapping clinical symptoms, particularly at early stages of disease.

Neuropathologically, PD and MSA are associated with abnormal aggregation of misfolded alpha-synuclein (α-syn) in the characteristic Lewy bodies of PD and the glial cytoplasmic inclusions (GCI's) of MSA. However, as a biomarker, body fluid levels of α-syn have shown limited utility in discriminating between PD and MSA although phosphorylated forms of the protein may have some potential in discriminating MSA from PD. DJ-1 and tau are implicated in the pathology of PD and have been shown to interact with α-syn, making them potentially interesting biomarkers.

DJ-1 (PARK7) is a small (~20kDa) multifunctional protein that functions primarily as an anti-oxidant and has roles in the modulation of anti-apoptotic and anti-inflammatory pathways. Mutations in the PARK7 gene result in familial autosomal recessive forms of PD. DJ-1 is abundantly expressed in reactive astrocytes of both PD and MSA. Tau functions in the stabilisation and regulation of microtubule assembly and these functions are regulated by its phosphorylation state. In several neurodegenerative diseases, tau is accumulated and/or hyperphosphorylated which likely plays a role in the pathogenesis of disease.

Immunohistochemical studies of DJ-1 and tau show that the GCI's of MSA are reactive for both DJ-1 and tau whereas the Lewy bodies of PD show only halo reactivity, or are only reactive for antibodies directed against phosphorylated tau. This suggests differing pathological roles of DJ-1 and tau in PD and MSA. Studies investigating CSF levels of DJ-1 and tau as biomarkers for PD and MSA are limited, and variable with some showing increased DJ-1 in PD compared with controls and others showing reduced DJ-1 in both MSA and PD compared with controls. In this study, we aimed to determine the utility of CSF DJ-1 and tau levels for discriminating MSA from PD and controls.

Methods
Patients and controls
Age and gender matched patients referred to our tertiary Movement Disorder Centre between January 2003 and December 2006 with a hypokinetic rigid syndrome of uncertain diagnosis at presentation, and who received a subsequent diagnosis of PD or MSA, were included in this study. Consenting patients underwent neurological examination, imaging analysis (MRI) and lumbar puncture (details provided as Supplementary data). Patients were re-examined after 3 years and final diagnosis was confirmed by case review at extended (3-9 year) follow-up. Disease severity was established as outlined in the Supplementary data. Medical ethical approval for the study was obtained from the Institutional Review Board.
Reference values were obtained from the CSF of patients referred to our Neurology Department in the period 2001-2009, who underwent lumbar puncture as part of the diagnostic process, and who were later confirmed as having no neurological disease. These controls included patients with subjective cognitive complaints not objectified by a neurologist (n=14); suspected of an (acute) neurological disorder which was not found (e.g. tension-type headache, lower back pain, etc.; n=4); without indication for a neurological disorder after investigation (n=6); depression or psychiatric problems (n=5); history of a cerebrovascular infarct (n=1).

**CSF collection, storage and analysis**

CSF samples obtained by lumbar puncture at the initial visit were collected in polypropylene tubes, transported to the laboratory, centrifuged (5min, 860x g at room temperature), transferred to clean polypropylene tubes and stored in aliquots at -80°C. Patient information was decoded to maintain patient confidentiality.

Measurements of DJ-1 in undiluted CSF were quantified using a commercially available sandwich format ELISA (IC DuoSet for PARK7/DJ-1; R&D Systems, Abingdon, UK) according to the manufacturer's general ELISA protocol. Internal controls were included to ensure consistency between ELISA plates. Measurements of total (t-tau) and phosphorylated tau (p-tau) were performed using the Innotest ELISA kits (INNOTEST® hTau Ag and phospho-tau(181p), Innogenetics N.V. Ghent, Belgium) according to the manufacturer's instructions. To examine correlations between DJ-1 and α-syn we used previously published α-syn levels for our PD and MSA patients 5. The ELISA method used for these α-syn levels is reported in the Supplementary data file.

**Statistical analysis**

Data showed a non-Gaussian distribution and was log-transformed prior to further analysis in order to meet the assumption of Gaussian distribution. Between-group differences were tested using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test or Student t-tests in the case of two-group comparisons. Pearson's correlation was used to test correlations between DJ-1, log-transformed tau, α-syn, hemoglobin (Hb), disease duration, and age. Binary logistic regression analysis and receiver operator curves (ROCs) were used to determine the diagnostic value of CSF parameters. All statistical analyses were performed using GraphPad PRISM 5 software (San Diego, California) or SPSS software version 20.0 (Chicago, IL). MedCalc® version 12.7.0.0 was used for ROC curve comparison.

**Results**

Forty-three patients with PD, 23 with MSA and 30 age- and gender-matched, non-neurological controls were included in the study. Patient characteristics and CSF parameters are provided in Table 1. Consistent with previous reports 20, we observed a strong correlation between DJ-1 and Hb >0.10 µmol/L (r = 0.65; p < 0.01) leading to falsely elevated DJ-1 levels. Although this did not alter the outcome (data not shown), we excluded all samples with a Hb ≥ 0.10 µmol/L (our lowest limit of detection) from further analysis to remove this confounder. This left 37 PD, 18 MSA and 23 control CSF samples for DJ-1 analysis. DJ-1 levels were significantly higher in MSA patients (710 ± 156 ng/L; Fig. 1) compared with PD patients (525 ± 117 ng/L; p < 0.001) and controls (418 ± 131 ng/L; p < 0.001), and significantly higher in PD patients than controls (p< 0.01). DJ-1 was not correlated with age, gender, disease duration or any measure of disease severity although
there was a trend towards higher DJ-1 with increased age in the PD patients ($r = 0.315, p = 0.062$).

T-tau levels were significantly reduced in PD (206 ± 116 ng/L) compared with MSA (283 ± 132 ng/L; $p < 0.001$) but not controls (241 ± 94 ng/L; $p = 0.11$). P-tau did not differ between the groups ($p = 0.35$). Age correlated with t-tau in the PD group and with p-tau in the controls. After controlling for age and gender, all significant differences reported above were maintained. Neither t-tau nor p-tau correlated with gender, disease duration, disease severity or MMSE.

DJ-1 discriminated MSA from controls with a sensitivity of 78% and a specificity of 100% (at a cut-off of $>0.615.0; AUC = 0.84$). DJ-1 could distinguish PD from controls at a cut-off of $>416.9$ and AUC = 0.71, with a sensitivity of 81% and specificity of 52%. Binary logistic regression with block entry using DJ-1, t-tau and p-tau levels from MSA and PD patients, provided a model (Model 1: $-8.409 + 0.018^{*}$DJ-1 + 0.024*$t$-tau - 0.184*$p$-tau) that significantly improved the ability to distinguish MSA from PD with a sensitivity of 82% specificity of 81% ($AUC = 0.92$; ROC curve comparison; $p < 0.05$). DJ-1 levels were not correlated with α-syn levels (Table S2) in either PD ($p = 0.33$) or MSA ($p = 0.107$). Adding α-syn to the model did not improve the discrimination between PD and MSA ($AUC = 0.92$).

**Discussion**

In the current study we assessed the utility of CSF levels of DJ-1, t-tau and p-tau in discriminating MSA from PD and controls. CSF levels of DJ-1 were significantly elevated in PD, and even more so in MSA, compared with controls. This allowed discrimination of MSA from both PD and controls with moderate to high sensitivity and specificity. Furthermore, the diagnostic accuracy for discriminating MSA from PD could be improved by combining measures of DJ-1 with t-tau and p-tau. Our results confirm earlier findings of elevated DJ-1 levels in PD patients 21 but the markedly elevated CSF DJ-1 levels in MSA in our study is unique and supports a role for DJ-1 in the pathogenesis of MSA.

**Table 1.** Patient characteristics and CSF DJ-1 and tau levels.

<table>
<thead>
<tr>
<th></th>
<th>PD</th>
<th>MSA</th>
<th>Control</th>
<th>p-Values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>43</td>
<td>23</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Gender: m/f (%)male</td>
<td>29/14 (67%)</td>
<td>14/9 (61%)</td>
<td>22/13 (63%)</td>
<td></td>
</tr>
<tr>
<td>Age at LP (yrs)</td>
<td>58.9 (9.8)</td>
<td>60.5 (7.5)</td>
<td>57.0 (11.7)</td>
<td>$p = 0.32$</td>
</tr>
<tr>
<td>Disease Duration (mths)</td>
<td>37.4 (30.9)</td>
<td>33.6 (21.6)</td>
<td>N/A</td>
<td>$p = 0.87$</td>
</tr>
<tr>
<td>Follow-up time: yrs (range)</td>
<td>4.6 (3 - 8.1)</td>
<td>3.7 (2.2 - 6.8)</td>
<td>N/A</td>
<td>$p = 0.09$</td>
</tr>
<tr>
<td>DJ-1 (ng/L)$^b$</td>
<td>525 (117), $n = 36$</td>
<td>710 (156), $n = 18$</td>
<td>418 (134), $n = 23$</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>t-tau (ng/L)$^c$</td>
<td>206 (116)</td>
<td>284 (131)</td>
<td>241 (94), $n = 23$</td>
<td>$p &lt; 0.01$</td>
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<tr>
<td>p-tau (ng/L)$^c$</td>
<td>46.7 (18.1)</td>
<td>49.3 (15.8)</td>
<td>52.3 (18.4), $n = 21$</td>
<td>$p = 0.35$</td>
</tr>
</tbody>
</table>

LP: lumbar puncture; N/A: not applicable.
* Significance using Student t-tests in the case of 2 groups, or one-way ANOVA with Tukey post-hoc test for 3-group comparisons.
$^b$ Number of cases remaining after removal of samples with Hb > 0.10 mmol/L (for DJ-1) is indicated in italic text.
$^c$ Not all controls samples had been tested for t-tau and p-tau; available numbers indicated in italic text.
Although DJ-1 levels are maintained at normal levels in neurons, they are up-regulated in reactive astrocytes in both PD and MSA, which may occur as a compensatory neuroprotective response to oxidative stress. Both DJ-1 and tau colocalise with α-syn in the GCIs of MSA but less so in PD Lewy bodies which are reactive to antibodies against phosphorylated tau but poorly reactive to phosphorylation-independent tau antibodies. This may provide a clue to the differing levels of CSF DJ-1 and tau in MSA and PD.

Our results contradict findings from another group in which lower levels of DJ-1 were observed in both MSA and PD compared with controls but support another study in which levels of DJ-1 in MSA were slightly, albeit non-significantly, higher compared with PD. While some studies support our finding of decreased t-tau in PD compared with MSA and controls, others show no differences between PD and controls. Comparison between individual studies is complicated by different methods and antibodies used to detect DJ-1 and tau proteins. This is supported by findings from an immuno-histochemical study which showed that various anti-DJ-1 antibodies reveal non-identical staining patterns for DJ-1 and suggests the presence of more than a single isoform of DJ-1. These DJ-1 proteins, in turn, may be differentially recognised in ELISAs using different combinations of antibodies.

Recent studies have shown that levels of α-syn are reduced in PD compared with controls but not compared to MSA. Previously, we described the absence of differences in α-syn levels between PD, MSA and controls and now we report the absence of a correlation between DJ-1 and α-syn in PD and MSA. Also, addition of CSF α-syn levels to our model did not improve the discrimination of PD from MSA. Recently it has been shown that differences between studies on CSF α-syn may arise due to the different platforms used in the different studies. Lack of suitable CSF refrained us from repeating the CSF α-syn analysis with a different type of assay. Therefore, additional studies investigating α-syn levels and correlations between α-syn and DJ-1 in PD, MSA and controls will be required to confirm our results.

Disparities in CSF parameters between studies could arise due to differences in control selection since we used non-neurological, but not necessarily healthy, controls whereas other studies used healthy controls or (other) neurological patients. Alternatively, age differences or possible neurological comorbidity in the various patient cohorts may have influenced results of the various studies. Furthermore, the extent of clinical follow-up may be a significant factor in assigning patients to a disease category. Indeed, an earlier study of a broader group of parkinsonism disorders with shorter follow-up than the current
study, reported that 30% of patients had changed diagnosis from inclusion to follow-up with most shifting from one parkinsonism disorder to another 22. Likewise, we observed that clinical diagnosis of some of our PD and MSA patients changed even after more than three years of follow-up. Therefore, misdiagnosis due to a more constricted follow-up may be a significant factor in these clinical studies. Although other previous studies on CSF DJ-1 reported longitudinal follow-up of controls with a median 3 year follow-up, the duration of follow-up for the PD and MSA patients was not specified, making it difficult to compare those results with ours 6,20. Regardless, we believe that combining a 3-year prospective study with extended case-review follow-up in each patient group is a major strength of our study and may have limited the rate of clinical misdiagnosis especially since previous studies have shown that differential diagnosis of PD from other atypical parkinsonism disorders after several years of follow-up correlates highly with neuropathological diagnosis upon post-mortem examination with misdiagnosis occurring mostly at initial diagnosis 22,34. Together, the high variation in DJ-1 levels between studies highlights the need for a validation and harmonisation of the methodologies used for the measurement of DJ-1.

In conclusion, our result show that levels of DJ-1 in CSF represent a possible biomarker for the differentiation of MSA from PD, particularly when used in combination with measures of t-tau and p-tau. Independent studies with clinical diagnoses after long-term follow-up or with neuropathological verification of the diagnosis are required to confirm these results and to determine whether levels of DJ-1 and/or tau proteins may also be able to discriminated MSA and PD from other atypical parkinsonisms.

Conflict of Interest
The authors declare no conflicts of interest with regard to the current study.

Funding sources
Financial support was obtained from the Van Alkemade-Keuls Fonds, The Netherlands.

Acknowledgements
We thank the technicians of the Department of Laboratory Medicine for performing the t-tau and p-tau analyses. This study was supported by research grants from the Van Alkemade Fonds and the Stichting Internationaal Parkinson Fonds. These Dutch non-profit funds had no role in study design or conduct of the study, data collection, data analysis or manuscript preparation.
Chapter 5

References


Supplementary Data

Supplementary Methods – Patients

Prospective, longitudinal analysis of patients

During the period September 2003 until November 2006, consecutive new patients with a hypokinetic rigid syndrome who had been referred to the movement disorders clinic of the Department of Neurology at the Radboud University Medical Centre in Nijmegen, the Netherlands were recruited for a three year prospective study. Inclusion and exclusion criteria are provided in Table M1. Patients diagnosed with Parkinson's disease (PD) and multiple system atrophy (MSA) were identified for inclusion in the study.

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**Supplementary Methods** - Alpha-synuclein ELISA method (van Geel et al., 2008 [13])

The ELISA assay is based on a previously described procedure with several important modifications [14]. A disposable flat-bottom microtiter plate (Nunc Maxisorp F96, Roskilde, Denmark) was coated with 100 µl antibody 211 (0.2µg/ml in 0.20M carbonate buffer pH 9.6) overnight at 4°C. A plate washer (BioTek, Beun de Ronde, Abcoude, the Netherlands) was used to wash the plate five times with 250µl PBS containing 0.05% Tween-20 (PBS washing buffer). All further incubations were performed at 37°C, unless stated otherwise, and all measurements were performed in duplicate. Two hundred and fifty microliters of blocking buffer (2.5% gelatin in PBS washing buffer) was added and incubated for 2 h and the plate was subsequently washed for five times with PBS washing buffer. Next, 100µl aSyn solution (from 0 to 500 ng/ml diluted in PBS) or CSF was added to each well and incubated for 2.5 h (in duplicate). Then, the plate was washed five times with PBS washing buffer and 100 µl of antibody FL-140, diluted 1:1000 in blocking buffer was added for 1.5 h. Again, the plate was washed five times and 100µl of the peroxidase labeled goat-anti rabbit antibody (dilution 1:5000 in blocking buffer) was added and incubated for 1 h. After a final washing step 100 µl of a freshly prepared solution of tetramethyl benzidine (TMB) was applied and incubated for 15 min in the dark at room temperature. The reaction was stopped by addition of 50 µl 2N H2SO4 and the absorbance was measured at 450 nm in a ELISA plate reader (Tecan Sunrise, Salzburg, Austria).
Supplementary Table S1

|                      | PD        | MSA       | α-syn Controls | p-values
|----------------------|-----------|-----------|----------------|---------
| **N**                | 43        | 23        | 57             |         |
| **Gender: m/f (% male)** | 29/14 (67%) | 14/9 (61%) | 30/27 (53%)    |         |
| **Age at LP (yrs)** | 58.9 (9.8)| 60.5 (7.5)| 61.3 (8.8)     | p = 0.43|
| **α-Syn (ng/mL)**   | 28.6 (12.8)| 26.3 (8.1)| 30.4 (19.1)    | p = 0.95|

* Control values as reported in Aerts et al., 2012 [15]; † Using ANOVA for 3-way comparisons and t-tests for 2-group comparisons; ‡ results reported as average (standard deviation).

Supplementary references

DJ-1 discriminates multiple system atrophy from Parkinson's disease
Chapter 6

Levels of HVA, 5-HIAA, and MHPG in the CSF of vascular parkinsonism compared to Parkinson’s disease and controls

Megan K Herbert, H Bea Kuiperij, Bastiaan R Bloem, Marcel M Verbeek

*Journal of Neurology* 2013; 260(12): 3129-3133.

"Well, no wonder you're late! Why this clock is EXACTLY two days slow!"
Abstract
The neurochemical abnormalities underlying vascular parkinsonism (VP) have not been well characterised. A better understanding may help to optimise pharmacological interventions. Since VP patients generally have a poorer response to L-Dopa than Parkinson's disease (PD) patients, we investigated whether levels of relevant CSF neurotransmitter metabolites may be differentially altered in VP and PD and assessed the potential of neurotransmitter metabolites as biomarkers. We compared CSF levels of homovanillic acid (HVA), 5-hydroxyindolacetic acid, and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), in 16 VP patients, 57 PD patients and 60 non-neurological controls. We found that levels of HVA were reduced in PD compared with both VP and controls but did not differ significantly between VP and controls indicating that dopamine deficiency was less pronounced in VP.
Introduction

Vascular parkinsonism (VP) arising from ischaemic cerebrovascular disease typically presents with lower body involvement, pyramidal signs and gait difficulties, and an absence of a resting tremor can help distinguish VP from Parkinson’s disease (PD)\(^1\). However, the pathophysiology of VP remains poorly understood and heterogeneous clinical symptoms most likely reflect variable underlying pathology\(^4\). Imaging studies (MRI and CT) frequently show small vessel disease (SVD) with diffuse white matter ischaemic lesions associated with multiple small lacunar infarcts (frequently occurring in basal ganglia or thalamus). The VP can also arise from subcortical arteriosclerotic encephalopathy (Binswanger’s disease) or a single infarct of the basal ganglia\(^2,5\). Symptoms often overlap with those of PD\(^4\) and, notably, the latter can give a clinical picture indistinguishable from PD, making clinical diagnosis particularly challenging.

While the most important neurochemical feature of PD is well characterised as a marked loss of dopamine\(^8\) and, to a lesser extent, serotonin (5-HT) and noradrenaline\(^9,10\), the extent to which these changes occur in VP is not well studied. Levodopa (L-Dopa), the first-line treatment for counteracting dopamine deficiency in PD, is also used to treat VP\(^1\). L-Dopa is efficacious in treating dopamine deficiency, but evidence for dopamine deficiency in VP is lacking. Although some VP patients (20-40%) respond to L-Dopa therapy, this is poor compared with PD responders (~80%), and treatment is often less effective and shorter-lived. We investigated CSF levels of homovanillic acid (HVA), 5-hydroxyindolacetic acid (5-HIAA), and 3-methoxy-4-hydroxyphenylethleneglycol (MHPG), the catabolic end products of dopamine, serotonin and adrenaline degradation, to determine whether these parameters are differentially altered in VP compared with PD and could represent potential biomarkers for distinguishing between VP and PD and controls.

Methods

Patients

Data from clinically defined VP and PD patients collected by the Department of Neurology and Laboratory Medicine at the Radboud University Medical Centre between May 1996 and December 2009, were retrospectively analysed together with data collected between 1993 and 2002 from non-neurological controls. Patients for whom lumbar puncture was performed as part of the diagnostic work-up and from whom informed consent was obtained, were included in the study. At presentation consenting patients underwent a detailed medical history, imaging studies, lumbar puncture and extensive neurological examination by a movement disorder specialist.

Clinical diagnosis was established according to current diagnostic criteria for PD\(^13\). The VP patients fulfilled all of the following inclusion criteria: older than 60 years, because older age at onset favours a vascular cause\(^6,14,15\); parkinsonism (such as rigidity and bradykinesia) predominantly of the lower body, with only mild involvement of the upper limbs\(^14,16\); a frontal gait disorder\(^6\); and neuroradiological evidence (CT scan) of vascular disease. Disease severity was established using the (modified) Hoehn & Yahr...
stages\textsuperscript{17} and the Unified Parkinson's Disease Rating Scale\textsuperscript{18}. Final diagnosis was confirmed by case review with clinical follow-up up to 8.5 years after initial visit.

We analysed data for CSF levels of HVA, 5-HIAA and MHPG. The CSF samples were collected in polypropylene tubes, centrifuged (5 min, 860 \( g \) at RT), and stored at -80°C. Patient information was decoded to maintain confidentiality. The HVA and 5-HIAA are dependent on the CSF fraction\textsuperscript{19}; thus only CSF samples from a separate 8th to 10th mL fraction were included. The methods of analysis of HVA 5-HIAA and MHPG in CSF have been previously described\textsuperscript{19,20}.

In brief, 1 mL CSF from this fraction was adjusted to pH 2.5 with formic acid and applied to a Sephadex G-10 (Sigma, St Louis, MO) column. After successive washing with formic acid and phosphate solution the metabolites were eluted with an ammonia solution into a tube containing both formic acid and ascorbic acid. High performance liquid chromatography (HPLC) was carried out using a mobile phase of phosphate-solution, citric acid and methanol on a Hypersil ODS-1 column (ThermoQuest, Breda, The Netherlands) with an amperometric detection.

The MHPG was measured by using the same method with some minor modifications. Namely, 0.5 mL of CSF was added to the column and this was successively washed with ammonia solution and formic acid, and eluted with 2 mL formic acid. The assays were linear within the following ranges: HVA, 0-4 \( \mu \)M; 5-HIAA, 0-2 \( \mu \)M; MHPG, 0-125 nM. Interassay variation coefficient was <4.8% in all three assays.

**Statistical Analysis**

Non-Gaussian distributed data was log transformed and between-groups analysis was performed using one way ANOVA with Tukey’s post hoc analysis and Pearson’s correlations. The CSF parameters were controlled for potential confounding factors (age and gender) using ANCOVA. All data were analysed using GraphPad Prism, version 5 and SPSS Statistical software, Version 20.

**Results**

Patient characteristics and CSF neurotransmitter metabolite levels from 57 PD patients, 16 VP patients and 52 control patients are shown in Table 1. Average age at time of lumbar puncture was significantly higher in the VP group (70.1 years) than the PD group (58.3 years; \( p < 0.0001 \)) but not compared with controls (62.4 years; \( p = 0.061 \)). Gender distribution was similar between the VP and PD groups (67 and 75% males, respectively). Although we had a lower percentage (48%) of males in the control group, the difference was not significant (\( \chi^2 = 5.64; p = 0.060 \)). The CSF levels of HVA, 5-HIAA and MHPG for the PD, VP and control groups are provided in Table 1 and Supplementary Figure S1.
HVA levels were significantly lower in PD (185 ± 108 nM) compared with VP (337 ± 384; p = 0.018) and showed a tendency towards lower values than controls (230 ± 106; p = 0.067). These differences were maintained after controlling for age and gender: PD versus VP (p = 0.017) and controls (p = 0.094). No differences in HVA levels were observed between VP and controls. Levels of 5-HIAA tended to be negatively correlated with age in PD (r = -0.26; p = 0.052) but not in VP (r = 0.26; p = 0.323) nor controls (r = 0.128; p = 0.365). Significantly lower levels of 5-HIAA were observed in PD (98 ± 40 nM) compared with controls (125 ± 53 nM; p = 0.010) and this was maintained after controlling for age and gender (p = 0.027). There were no significant differences in levels of 5-HIAA between VP and PD (p = 0.247) although there was a trend towards lower 5-HIAA levels in PD compared with VP after controlling for age and gender (p = 0.072). No significant differences in MHPG levels were found between any groups before or after controlling for confounding factors.

The CSF neurotransmitter levels in L-Dopa medicated versus unmedicated or non-L-Dopa medicated (together referred to as non-L-Dopa) patients are provided in Supplementary Table S1. L-Dopa treated VP patients (n = 8) had higher levels of HVA than L-Dopa medicated PD patients, even exceeding the maximum concentration (372 nM) of our normal range. L-Dopa treated VP patients had higher levels of HVA than non-L-Dopa treated PD patients and non-L-Dopa treated VP patients, suggesting that L-Dopa treatment boosts dopamine levels, as expected. Levels of 5-HIAA were significantly lower in the L-Dopa medicated PD patients (70 ± 33 nM) compared with both the L-Dopa medicated VP patients (117 ± 34 nM, p < 0.001) and non-L-Dopa PD patients (105 ± 38 nM; p < 0.01), but no differences in 5-HIAA between L-Dopa and non-L-Dopa medicated VP patients were observed. No significant differences in MHPG levels were detected for PD and VP or for medicated versus non-medicated analyses.
We observed a trend ($p = 0.09$) towards longer disease duration in VP (48 ± 35 months; $n = 14$) than PD (35 ± 29 months; $n = 53$) and disease duration tended to be correlated with HVA levels in PD ($r = 0.259; p = 0.061$) and negatively correlated with HVA in VP ($r = -0.462; p = 0.096$). The HVA levels were negatively correlated with H&Y scores in the PD group ($r = -0.351, p < 0.05$), but no other correlations between disease severity (UPDRS or H&Y) were found, regardless of medication status (data not shown).

**Discussion**

Analysis of monoamine neurotransmitter metabolites in CSF revealed a significant reduction of HVA in the CSF of PD compared with both VP and controls after controlling for age and gender. The significantly lower level of HVA in PD compared with VP is consistent with another, similar study $^{21}$. These results suggest that dopamine is more depleted in PD than VP. To our knowledge, we are the first to use correct CSF fractions $^{19}$ for the measurement of these neurotransmitter metabolites for comparison between VP and PD. Differences in HVA levels were not useful as a biomarker to distinguish VP patients from PD patients, but animal studies show decreased CSF HVA levels only when striatal lesions cause 50% or greater loss of dopamine $^{22}$, so this may not be unexpected.

We observed rather high heterogeneity in the levels of neurotransmitter metabolites in all groups. This could be partly due to the correlation of age and gender with some of the CSF parameters observed in the current study. In addition, other as yet unknown external factors may influence the results. For example, it has previously been shown that seasonal changes, circadian rhythms, levels of cognition and glucose may all have an effect on CSF levels of neurotransmitter metabolites $^{23-26}$. Several earlier papers have shown that dopamine levels are elevated in patients with chronic ischaemic damage (discussed in $^{21}$). Theoretically, this could subsequently lead to alterations in levels of its metabolite, HVA. Therefore, heterogeneity may have arisen due to comorbid ischaemic damage in the PD patients and controls, particularly given that our control group were 'non-neurological controls' rather than 'healthy controls' thus increasing the possibility of including controls with underlying ischaemic damage. In contrast, the heterogeneity seen among VP patients was not entirely unexpected as there is already a high degree of heterogeneity among these patients with regard to the location and degree of underlying pathology that likely contributed to the variability in HVA levels in these patients.

Recent studies show a relationship between white matter lesions and parkinsonism in patients with both mild parkinsonian signs $^{25}$ and vascular parkinsonism $^{4}$, particularly when vascular damage occurs in relevant brain regions such as basal ganglia-thalamocortical circuits $^{25}$. Thus, vascular damage to white matter tracts may contribute to parkinsonism symptoms even when dopaminergic nuclei are relatively preserved. Animal model studies of corpus striatum showed large increases in production of deaminated catecholamine metabolites during reperfusion following ischaemic assault and indicated that catecholamines may be released in large amounts during initial ischaemic insult $^{26}$. This could also explain why CSF HVA levels were higher in VP compared with PD and controls. This is also supported by other studies, which found higher levels of dopamine in patients with VP compared with controls that may account for the increase in the dopamine metabolite (HVA) levels (as discussed in $^{21}$).
L-Dopa is prescribed for the purpose of combating dopamine loss. The HVA levels in our VP patients, regardless of medication status, were not different from controls, and may partly explain limited responsiveness to L-Dopa in VP. These findings are supported by other studies showing that VP patients with lesion affecting areas rich in dopaminergic-producing neurons, such as the nigrostriatal pathway or substantia nigra, tend to respond better to L-Dopa therapy than patients with lesions in other brain areas not involved in dopamine release \(^{27}\). Furthermore, VP patients with normal uptake on striatal DAT, and, thus, relatively normal dopamine uptake, do not benefit from L-Dopa medication \(^4\). Taken together these studies suggest that dopamine depletion may occur in VP patients only when specific brain areas affecting dopamine production are damaged, and response to L-Dopa therapy may be higher in this group. This could also account for some of the high variability in L-Dopa responsiveness among VP patients reported in the literature (20 and 78%) \(^{14,28}\), and may reflect extensive variability in underlying pathology.

We observed significantly reduced 5-HIAA levels in PD compared with controls. These findings are consistent with findings of serotonergic disturbances in PD, particularly in median raphe and caudate nucleus \(^9,29\). Others suggest that L-Dopa treatment stimulates serotonergic neurons to preferentially release dopamine and, thus, reduce serotonin release \(^{30,31}\) so reduced 5-HIAA levels in CSF might be expected to reflect L-Dopa treatment. Indeed, L-Dopa treated PD patients had lower levels of 5-HIAA than non-L-Dopa treated PD patients (Supplementary Table S1). However, 5-HIAA levels were also reduced in the L-Dopa treated PD patients compared to the L-Dopa treated VP patients and controls. Therefore, reduced 5-HIAA levels in PD patients could also partly reflect serotonergic deficits rather than effects induced by L-Dopa. Although others showed reduced 5-HIAA levels in the brains and CSF of VP compared with PD, the CSF fraction used for analysis was not reported \(^21\) and may not have accurately reflected true CSF levels of 5-HIAA.

In conclusion, modestly higher concentrations of HVA in the CSF of VP compared with PD patients may indicate more severe dopaminergic cell loss in PD and may partly clarify why VP patients have a poorer L-Dopa responsiveness. We would expect that patients with lower levels of HVA may respond more positively to L-Dopa medications than those with normal levels. Much larger patient groups with (eventual) neuropathological confirmation of disease will be required to clarify our results and to elucidate the relationship between neurotransmitter metabolites levels and underlying pathophysiology of vascular parkinsonism.

**Acknowledgements**

We thank the technicians of the Department of Laboratory Medicine (LGEM) for CSF analysis.
Supplementary Data

Supplementary Table S1. Neurotransmitter metabolite levels according to patient medication

<table>
<thead>
<tr>
<th></th>
<th>HVA (nM) mean (s.d.)</th>
<th>5-HIAA (nM) mean (s.d.)</th>
<th>MHPG (nM) mean (s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non L-Dopa medications; n=44</td>
<td>170 (73)</td>
<td>105 (38)</td>
<td>47 (9)</td>
</tr>
<tr>
<td>L-Dopa; n=12</td>
<td>233 (187)</td>
<td>70 (33)</td>
<td>47 (17)</td>
</tr>
<tr>
<td>VP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non L-Dopa medications; n=8</td>
<td>207 (121)</td>
<td>121 (57)</td>
<td>46 (14)</td>
</tr>
<tr>
<td>L-Dopa; n=8</td>
<td>506 (502)</td>
<td>117 (34)</td>
<td>58 (17)</td>
</tr>
</tbody>
</table>

*Medication status was unknown for 1 PD and 1 VP patient and are not included in the analysis.

Supplementary Figure S1

Figure S1. Levels of neurotransmitters metabolites in CSF. CSF 5-HIAA (A) and HVA (B) levels were higher in VP patients compared with PD patients but no differences in MHPG levels (C) were observed.
**References**


CSF neurotransmitter metabolites in parkinsonism disorders
Demyelinating disorders such as multiple sclerosis are characterised by nervous system damage that is disseminated in time and place. The photo series in part 4 is intended to represent the recurrence of disease at different places and at different times.
Part 4

The use of CSF biomarkers for the differential diagnosis of demyelinating disorders

“How puzzling all these changes are! I’m never sure what I’m going to be, from one minute to another.”
Chapter 7

Optimisation of the quantification of glutamine synthetase and myelin basic protein in cerebrospinal fluid by a combined acidification and neutralisation protocol.

Megan K Herbert, H Bea Kuiperij, Marcel M Verbeek

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“I know who I was when I got up this morning, but I think I must have changed several times since then”
Abstract

The measurement of proteins in cerebrospinal fluid (CSF) by enzyme-linked immunosorbent assays (ELISAs) is becoming increasingly important in the diagnosis of many neurodegenerative diseases such as Alzheimer’s Disease. However, detection of proteins in these immunoassays can be hampered by confounding factors either present in the sample matrix or inherent to the protein of interest. These confounding factors may, for example, include protein aggregation or binding to other proteins resulting in epitope masking. Furthermore, the pH of CSF may vary considerably amongst different samples which may limit standardisation of CSF analysis. Pretreatment of CSF to liberate epitopes or optimise conditions for antibody binding may enhance protein detection. In the current study we investigated whether CSF acidification followed by neutralisation (in short: AFBN) or neutralisation alone prior to measurement might improve the detection of a panel of brain-specific proteins. We demonstrate that the AFBN pre-treatment protocol for CSF significantly enhances the measurement of glutamine synthetase (GS) and myelin basic protein (MBP) in CSF but does not affect detection of glial fibrillary protein (GFAP), amyloid β 42 (Aβ42), total tau (t-tau) or phosphorylated tau (p-tau). Neutralisation alone did not improve detection of any of the proteins tested. Based on our results, we suggest including the AFBN protocol in the evaluation of new biomarker development protocols to avoid confounders such as CSF pH or epitope-masking of the target protein.
Introduction

The quantification of proteins in CSF is becoming increasingly important in the diagnosis of neurodegenerative diseases such as Alzheimer's Disease (AD) (Dubois et al., 2007; McKhann et al., 2011). Changes in the levels of brain-specific proteins in CSF have been shown to be associated with disease pathogenesis and brain dysfunction (Verbeek et al., 2003; Olsson et al., 2011). Proteins of interest for AD include amyloid beta protein (Aβ42), total and phosphorylated tau protein, and glutamine synthetase (GS). Aβ42 and tau are well known to aggregate and deposit in the brain to form extracellular senile plaques and intra-neuronal fibrillary tangles respectively and are considered to be the neuropathological hallmarks of AD (Ittner and Götz, 2011). GS, an enzyme which converts potentially toxic glutamate and ammonia to non-toxic glutamine, is also of interest as its activity is reduced in the brains of patients with AD and may thus contribute to increased neuronal excitotoxicity (Prince et al., 1995; Olabarria et al., 2011).

CSF proteins can be measured by relatively simple, efficient methods. Sandwich-format enzyme-linked immunosorbent assays (sELISAs) are a commonly used platform for sensitive and specific measurement of proteins in CSF and several commercially available kits have shown high utility in the diagnosis of specific diseases. Indeed, the commercially available kits for the measurement of brain specific proteins total tau, (t-tau), phosphorylated tau (p-tau) and amyloid β42 protein (Aβ42) in CSF are currently being considered for addition to diagnostic criteria for AD (Mattsson et al., 2009; Mattsson, 2011).

However, accurate measurement of CSF proteins by ELISA can be adversely affected by extraneous factors. Those that have been shown to influence the detection of t-tau, p-tau and Aβ42 include CSF storage conditions, adsorption of CSF proteins to collection tubes, and batch-to-batch variations in ELISA kits, leading to inconsistencies in reference ranges and clinical characteristics of the assays between analytical centres (Bjerke et al., 2010). As a result, recommendations for the standardisation of CSF collection have been suggested (Bjerke et al., 2010; Teunissen et al., 2010) and, under the auspices of the American Alzheimer Association, a harmonisation process for the (pre-) analytical analysis of these biomarkers has been started.

In addition to the aforementioned factors, other confounding factors inherent to the protein of interest or to the CSF sample matrix may lead to reduced immunological detection by individual antibodies (Hnasko et al., 2011). The binding of other substances (e.g. autoantibodies, lipids, or other proteins) may cause a change in protein conformation that alters epitope conformation and/or ‘masks’ the epitope thus preventing the binding of individual antibodies (Okragly and Haak-Frendscho, 1997; Bibl et al., 2004). This epitope masking by binding substances has been suggested as one of the factors underlying the relative large variability in the quantification of brain-specific proteins in CSF (Bjerke et al., 2010). Furthermore, the specificity or epitope binding of commercial antibodies is not generally well-characterised and the optimal conditions for antibody binding in ELISA must be examined for individual antibodies (Hnasko et al., 2011).

In the past, several pre-treatment methods for CSF have been considered for improved detection of proteins in ELISA. These included detergent treatments (e.g. SDS, Tween-20) and heat denaturation of proteins (Groome et al., 1986; Bibl et al., 2004). However, repeated measures showed inconsistencies, possibly due to other types of interference. For example, the presence of SDS at high concentrations can interfere with ELISA assays
by introducing non-specific antibody binding (Julian et al., 2001) whereas applying low concentrations of SDS may be insufficient to fully denature proteins for improved detection (Hnasko et al., 2011).

An alternative pre-treatment of CSF for epitope unmasking involves the acidification of CSF followed by neutralisation prior to analysis (Lyons et al., 1988). The detection of the active form of transforming growth factor-β (TGF-β) requires this step to enable release from its proform (Lyons et al., 1988). Myelin basic protein (MBP) is an essential component of myelin that is degraded in multiple sclerosis (MS) and high CSF levels of the protein may indicate acute exacerbation or progression of disease (Lamers et al., 1998). Similarly, MBP autoantibodies have been investigated for their role in nerve demyelination, a key pathological feature in MS (Belogurov et al., 2008). However, sandwich ELISAs for the detection of MBP and MBP autoantibodies seem to be affected by epitope masking. Hence an acid pre-treatment of CSF to release MBP auto-antibodies that are bound to the MBP protein are often incorporated into sELISA protocols (Warren and Catz, 1987; Mastroianni et al., 1991). This also suggests that epitope-masking may play a role in the quantification of MBP itself in CSF.

Other proteins tested using an acidification followed by neutralisation (AFBN) pre-treatment of CSF include glial cell derived neurotrophic factor (GDNF), cyclic guanidine 5'-monophosphate (cGMP) and nerve growth factor (NGF), where improved protein detection was also observed (Lyons et al., 1988; Zettler et al., 1996; Okragly and Haak-Frendscho, 1997; Grundström et al., 2000; Iłżecka, 2004). The mechanism of action of AFBN-enhanced detection of proteins such as GDNF and NGF may be a result of the irreversible dissociation of target proteins from other proteins, or substrates, or through the disassembly of larger multimeric forms of the protein into smaller oligomeric and monomeric forms (Lyons et al., 1988; Zettler et al., 1996; Okragly and Haak-Frendscho, 1997; Bibl et al., 2004).

In this study we investigated whether AFBN pre-treatment would improve the detection of several brain-specific proteins, including MBP, GS, glial fibrillary acidic protein (GFAP), Aβ42, t-tau and p-tau, to study if the detection of these proteins is hindered by binding substances. In addition, since naïve CSF may have a very high pH, we determined whether simply adjusting the CSF pH to physiological levels (pH 7.0 to 7.4) prior to measurement would improve the standardisation of CSF protein analysis by ELISA.

**Methods**

**CSF samples**

CSF samples were obtained by lumbar puncture and collected in polypropylene tubes followed by 5 min of centrifugation (860 ×g at room temperature) within 2 h after collection. Following centrifugation, samples were transferred to clean polypropylene tubes. Samples were stored at −80 °C until day of testing and no sample had more than one freeze/thaw cycle prior to testing. Separate aliquots taken from the same sample were used for assessing the effects of pH on the measurement of proteins in one and the same ELISA. All patient information was decoded to maintain patient confidentiality. Informed consent was obtained from the patient or his legal representative.
Neutralisation of CSF samples
Aliquots of CSF for all ELISAs, except for GS, were diluted in 250 mM potassium phosphate buffer (PPB), pH 7.2 at a ratio of 4:1 in PPB to adjust the CSF pH to neutral. For GS ELISAs, aliquots of CSF were diluted 1:1 with 0.1% BSA in 50 mM PPB, pH 7.8 to adjust the pH to neutral. pH measurements were performed using a standard pH meter (PHM210 Meterlab, Radiometer Analytical, France).

Pre-treatment of CSF samples using an acidification followed by neutralisation (AFBN) protocol
An aliquot of each CSF was acidified to pH ≤3 by the addition of 4.8 μL 2M HCl per 120 μL of CSF and incubated for 10 min at room temperature (RT). Samples were neutralised by the addition of 3.2 μL 2M NaOH and 250 mM PPB (pH 8.4) to give a final CSF:PPB ratio of 4:1. In the case of GS, 0.1% BSA in 50 mM PPB, pH 7.8 was added at a ratio of 1:1 after addition of NaOH, in order to neutralise the samples.

Protein expression and purification of GS
GS with an N-terminal His-tag (His-GS) was expressed in Escherichia coli cells followed by purification under native conditions using a Nickel Nitrilotriacetic acid (Ni-NTA) chromatography column (Qiagen, Hilden, Germany). The purified protein was transferred to a Slide-A-Lyzer cassette (12–30 mL, MWCO 10 K, Thermo Scientific, Rockford, IL, USA) and dialysed in native lysis buffer (NLB; 50 mM NaH₂PO₄, 300 mM NaCl, pH 8) to reduce the imidazole content. The dialyzed protein was concentrated in the dialysis cassette using solid polyethylene glycol (PEG; 20–35 kDa), MnCl₂, and ATP (final concentration of 1 mM each) were added to a separate cassette containing dialyzed protein to help stabilise the protein (Krajewski et al., 2008) prior to concentration using a 9 kDa cut-off Pierce® protein concentrator (Thermo Scientific). The final protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA).

CSF ELISAs
Undiluted, neutralised and AFBN treated CSF samples were tested for GS, MBP, GFAP, t-tau, p-tau, and Aβ42 as follows:

Sandwich ELISA for GS
A homemade sandwich ELISA using His-GS for the standard curve was set up for the measurement of GS. Wells of a 96-well flat-bottom ELISA plate (F96 cert. MaxiSorp™ Immuno-plate: Nunc, Roskilde, Denmark) were coated with monoclonal mouse antibody directed against human GS (Abnova, Taipei City, Taiwan) at 0.05 μg/mL in coating buffer (PBS, pH 7.2-7.4) and incubated overnight (O/N) at 4 °C. Plates were washed 5 times with 300 μL of 0.05% Tween-20 in phosphate buffered saline (PBS) between all incubation steps. The plate was blocked with 250 μL/well of 1% BSA in 0.1 M PPB, pH 7.8 and incubation at RT for 1 h. All subsequent incubations were performed at 37 °C with agitation at 650 rpm. First, 100 μL of standards, blanks (0.1% BSA in 50 mM PPB, pH 7.8) plus untreated and pre-treated CSF samples were incubated for 2 h. Wells were then incubated with 100 μL of polyclonal rabbit antibody directed against human GS (0.3 μg/mL; Proteintech Group, Chicago, IL, USA) in antibody buffer (AbB1; 0.05% Tween-20/0.1% BSA in 50 mM PPB) for 1 h. The secondary antibody, 100 μL per well of biotinylated goat anti-rabbit antibody (Dako, Glostrup, Denmark; 1:3000 in AbB1), was incubated for 1 h followed by a subsequent incubation with 100 μL of streptavidin-HRP complex (Life Technologies,
Camarillo, CA, USA; 1:60,000 in AbB1) for 30 min. The colour reaction was developed using 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide incubated for 10–30 min at RT, in the dark. The reaction was stopped using 0.5 M sulphuric acid and the plate was read at 450 nm using the Tecan Sunrise ELISA plate reader and Magellan program.

**MBP sandwich ELISA**

MBP analysis was performed using a homemade sandwich ELISA in which wells of an ELISA plate were coated O/N at 4 °C with a mixture of mouse monoclonal antibody directed against MBP (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal rat anti-MBP (1:500 Millipore, Billerica, CA, USA) diluted in coating buffer (0.1 M NaHCO₃, pH 9.5). All subsequent incubations were performed at RT. Washing was performed between each incubation step using five 300 μL washes of PBST and plates were blocked with 1%BSA/PBS for 1 h. Purified human MBP (a gift from Dr Sindic, Leuven, Belgium) in the range 0.1 to 6.25 μg/L (used as standards) or pre-treated, neutralised and untreated CSF samples (diluted 1:1 in AbB2; 1% BSA/0.05% Tween-20/sodium phosphate buffer (64.6 mM NaHPO₄·2H₂O; 21 mM NaH₂PO₄·H₂O, pH 7.4) were added to wells and incubated for 2 h at 25 °C. Polyclonal rabbit anti-human-MBP (1:3000 in AbB2; Dako) was added with incubation for 1 h at 25 °C. After removing unbound antibody, HRP-conjugated goat anti-rabbit antibody (1:5000 in AbB2; Jackson, Bar Harbour, ME, USA) was added. The colour reaction and data collection were performed as for the GS sELISA.

**Sandwich ELISAs for GFAP, Aβ42 and tau**

GFAP was quantified using a homemade sandwich ELISA (linear up to 250 μg/L; interassay variation coefficient <14%) as described previously (van Geel et al., 2002; Abdo et al., 2004). Aβ42, t-tau, and p-tau concentrations were determined using commercially available ELISA kits (INNOTEST®, Innogenetics N.V., Ghent, Belgium). All assays were performed according to manufacturer’s instructions. However, for the measurement of t-tau we included additional standards with concentrations 1200 pg/mL and 2400 pg/mL.

**GS Depletion experiments**

Aliquots of AFBN-treated and neutralised CSF were incubated with monoclonal mouse antibody (IgG) directed against GS (final concentration of 0.5 μg/mL) for 1 h at RT with gentle rolling. Samples were added to pre-washed Protein G agarose beads and incubated for a further 1 h with gentle rolling, to allow binding of the GS/anti-GS-IgG antibody complex. Finally, GS-depleted sample was obtained by centrifugation for 1 min at 5200 rpm before its use in ELISA.

**Analysis of decameric and monomeric forms of GS using transglutaminase cross-linking and western blot analysis**

His-GS proteins were crosslinked using transglutaminase from guinea pig liver (TG) as follows: a mixture of 16 μL 2X cross-linking buffer (0.1 M Tris–HCl, 300 mM NaCl, 30 mM DTT and 40% glycerol, pH 7.5) was pre-incubated with 3.2 μL of 1 mg/mL TG (Sigma, St Louis, MO, USA) for 15 min at 37 °C to activate the enzyme (Boros et al., 2004). Aliquots containing 3.5 μg of His-GS protein were added to the cross-linking mixture together with 3.2 μL of 50 mM CaCl₂, in a final volume of 32 μL, and incubated for a further 2 h at 37 °C. Immediately following cross-linking, 10 μL of 4X reducing sample buffer (25% (w/v) glycerol, 2% (w/v) SDS, 62.5 mM Tris–HCl, pH 6.8, 32 mM DTT and 0.005% (w/v) bromophenol blue)were added to the mixture. Aliquots were heated to 95 °C for 5 min and loaded onto a 12% SDS-PAGE gel. The gel was electrophoresed in running buffer (25
mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 200 V for 1 h. Proteins were transferred to PVDF membranes for 1 h at 100 V in transfer buffer (2.5 mM Tris, 19.2 mM glycine, 20% methanol). The membrane was blocked with blocking buffer (1:1 PBS: Odyssey blocking buffer (LiCor, Lincoln, Nebraska, USA)) followed by an O/N incubation at 4 °C with polyclonal rabbit anti-GS (1:1000) in blocking buffer. After 5 washes with PBST, the membrane was then incubated for 1 h at RT with goat anti-rabbit Alexa 680 (1:5000; Life Technologies) in blocking buffer. Fluorescence was visualised using an Odyssey scanner at 700 nm. The membrane was then re-incubated O/N at 4 °C with monoclonal mouse anti-GS (1:1000) and, following washing, further incubated with goat anti-mouse IRDye® 800 (1:5000; LiCor) in blocking buffer. The membrane was then rescanned at 800 nm.

Statistics

GraphPad PRISM 5 software (San Diego, California) was used for statistical analysis. Comparison of differences in CSF protein concentrations between untreated and treated (AFBN or neutralisation only) samples was performed using paired t-test analysis. The Spearman rank correlation was used for correlations.

Results

CSF pH analysis following AFBN or neutralisation alone

Prior to treatment, the pH of CSF samples varied from 7.7 to 9.2 (mean pH 8.5, standard deviation (SD) 0.4; Fig. 1A and B). After applying the GS neutralisation protocol (Fig. 1A) the mean pH was 7.3 (SD 0.15; n=10) and, after AFBN, the final pH was 7.3 (SD 0.11; n=40). The neutralisation protocol for all other proteins (Fig. 1B) resulted in a mean pH of 7.2 (SD 0.06; n=40), whereas AFBN treatment resulted in a mean pH of 7.3 (SD 0.21; n=40).

Detection of GS in CSF requires AFBN pre-treatment

GS was rarely detectable in either untreated or neutralised CSF samples but could be detected in 73% of AFBN treated CSF samples, with a mean concentration of 0.21 μg/mL (SD 0.06) (Fig. 2). A typical standard curve for GS can be seen in Fig. 3. The assay is linear between 0.06 and 1 μg/mL (r²=0.9975). The lower detection limit of the assay was 0.019 μg/mL.

Figure 1. Final pH values for untreated, neutralised and AFBN-treated CSF samples. Figure A shows the pH Values obtained following neutralisation and AFBN treatment following the GS protocol. Figure B illustrates the values obtained following the neutralisation and AFBM treatments used for all other proteins. Boxes represent median values with upper and lower quartiles. Whiskers represent minimal and maximal values.
To determine the specificity of the assay, GS was depleted from CSF. This resulted in a reduction of GS concentration of up to 80% in AFBN-treated samples, suggesting that the ELISA was indeed specific for the detection of GS in CSF.

**Analysis of decameric and monomeric forms of GS using TG cross-linking and western blot analysis**

The increased detection of GS after AFBN treatment may be caused by release of individual GS units from the decameric protein complex. In order to ensure that decameric GS would remain in this form under the reducing conditions of SDS-PAGE gel, we cross-linked the protein with TG, to stabilise the decameric form of GS without affecting the monomeric form of GS. Our results showed that the His-GS purified in the absence of Mn/ATP was predominantly present as monomers and this was relatively unchanged following the cross-linking procedure (Fig. 4). In stark contrast, the His-GS purified in the presence of Mn/ATP and cross-linked thereafter remained predominantly in the stacking gel even under reducing conditions. This suggests that it is predominantly present in a high molecular weight (decameric) form in contrast to the non-cross-linked form which was predominantly present as monomers. In line with this finding, and as seen with CSF samples, the (decameric) His-GS purified in the presence of Mn/ATP was not detectable in the sELISA unless first treated using the AFBN protocol (data not shown).

**AFBN treatment improves MBP, but not GFAP, detection in CSF**

Detection levels for MBP were significantly improved by the AFBN protocol with an average 50% increase in signal ($p<0.005$) (Fig. 5). Neutralisation alone did not confer an effect on MBP quantification, indicating that the acidification step of the AFBN protocol is necessary for the signal increase. CSF levels of GFAP were significantly lowered by an average 32% by the buffer neutralisation of CSF prior to analysis ($p<0.05$) and the AFBN protocol conferred no significant change in the levels of GFAP detected in CSF (Fig. 6).

**Quantification of Aβ42, t-tau and p-tau is significantly reduced by pre-treatment of CSF**

There was a small but consistent and significant reduction in measured levels of Aβ42, t-tau and p-tau following both neutralisation alone and AFBN ($p<0.05$ in all cases; Fig. 7). In the case of Aβ42, neutralisation alone caused a greater reduction (~25%) than that for AFBN (~11%) (Fig. 7A). Levels of t-tau (Fig. 7B) were reduced both by neutralisation alone (~9%) and by AFBN (~14%). Finally, levels of p-tau (Fig. 7C) were equally reduced by either neutralisation alone (~11%) or AFBN (~10%).

Fig. 2. GS levels of untreated and AFBN pre-treated CSF samples. GS levels were measured by sELISA in CSF (n=7).
Fig. 3. Standard curve for GS in the range 0.06 μg/mL to 1 μg/mL. His-GS: Histagged recombinant human glutamine synthetase; OD450: optical density at 450 nm.

Fig. 4. Transglutaminase (TG) cross-linking of His-GS purified in the presence or absence of Mn/ATP. Pre- and post-TG cross-linked His-GS (with or without Mn/ATP; 3.5 μg) were separated by SDS-PAGE and immunoblotted with an antibody directed against GS. Prior to cross-linking, both the His-GS without Mn/ATP (lane 1) and the His-GS with Mn/ATP (lane 3) were predominantly present in a monomeric form. Following cross-linking, the His-GS without Mn/ATP (lane 2) retained its predominantly monomeric form whereas the His-GS with Mn/ATP (lane 4) can be seen in a predominantly decameric form.

Fig. 5. Effect of neutralisation (CSF(N)) and AFBN treatment (CSF(A)) on the measurement of MBP in CSF (n=18). Results for untreated CSF are shown as “CSF”. Bars indicate standard error of the mean. ns=non-significant, **=p<0.01.
Chapter 7

Discussion
Numerous extrinsic and intrinsic factors can confound the identification and measurement of proteins in biological fluids, prohibiting standardisation of analyses and explaining the observed discrepancies in inter- and intra-laboratory measurements of CSF proteins (Hühmer et al., 2006; Bjerke et al., 2010). Variation in CSF pH and epitope masking may be such confounders and were investigated in our study. Our main findings are: 1) An AFBN pre-treatment of CSF can be used to improve the detection of some brain-specific proteins such as MBP and GS, likely by improving antigen–antibody interactions and 2) Neutralisation alone of CSF prior to analysis by sELISA could not be shown to confer improved protein detection but rather leads to impaired detection of most proteins (i.e. GFAP, Aβ42, t-tau and p-tau).

Antibody-based detection of proteins in CSF is influenced by the accessibility of antibody-specific epitopes of the target protein and by the conformation of the epitope for antibody binding. Thus the improvement we observed in GS and MBP detection after AFBN is most likely due to unmasking of hidden epitopes or alterations in epitope conformation that facilitate improved antigen–antibody interactions and binding. Factors that can contribute to epitope-masking include the association of proteins with other binding proteins (e.g. lipids, carrier proteins, auto-antibodies), self-aggregation of the target protein (e.g. in the case of aggregating proteins like Aβ and tau) (Groome et al., 1986; Okragly and Haak-Frendscho, 1997), or the secretion/release of proteins from a latent proform (Douglas et al., 2009). Various techniques have been utilised to facilitate the unmasking of epitopes including detergent or heat denaturation (Lechtzier et al., 2002; Bibl et al., 2004; Bjerke et al., 2010) and pre-treatment of samples with acid (Groome et al., 1986; Okragly and Haak-Frendscho, 1997).

It has previously been demonstrated that the detection of MBP auto-antibodies in CSF can be improved by pre-treatment with AFBN (Groome et al., 1986; Mastroianni et al., 1991). Thus, the improved detection of MBP in the AFBN-treated CSF tested in our sandwich ELISA likely arises from the release of MBP autoantibodies from antigen-antibody complexes. This would release the MBP epitopes to facilitate binding of the capture and detection antibodies used in the sandwich ELISA format. Alternatively, the binding of lipids or other proteins to MBP may lead to epitope masking, as has been previously suggested (Macklin et al., 1981), and the acidification of CSF may release these lipids binding to MBP protein thus exposing otherwise hidden epitopes. Further investigation is required to determine whether the improved detection of MBP in CSF can also improve its utility as a biomarker for MS.

A recent study demonstrated that the detection of GS by indirect ELISA format (iELISA) was improved by denaturation of the protein most likely due to a conformational change in the protein that enhanced the binding of the protein to the ELISA plate (Hnasko et al., 2011). Since in our study AFBN treatment was essential for enabling detection of GS in CSF samples, we performed additional experiments with cross-linking and western blotting to further explore the possible mechanisms of action. It is known that GS may occur both in a monomeric and decameric form and that the latter is stabilised by the presence of Mn and ATP (Krajewski et al., 2008). We demonstrate that different conformations of recombinant His-tagged GS are possible and our findings suggest that the improved detection of GS in CSF by sandwich ELISA following AFBN treatment may arise from a conformation change in the protein. In this case, a likely conformational change from a (decameric) native form
of the protein to a smaller, monomeric form improved the detection of the protein in our sandwich ELISA.

GS concentrated in the absence of Mn and ATP appeared predominantly in a monomeric form whereas the GS concentrated in the presence of Mn and ATP was predominantly present in a multimeric form which more closely reflects the expected physiological form of the protein. Furthermore, the monomeric form (obtained without AFBN treatment) was used to produce a standard with good linear fit for the sELISA. Thus AFBN treatment most likely reduces the native (decameric) GS in CSF to a monomeric form that could then be detected by the sELISA. Previously developed sELISAs which use a single monoclonal antibody for both capture and detection of GS in CSF are capable only of measuring decamers of the protein (Tumani et al., 1995; Tumani et al., 1999). While these sELISAs identified an increase in GS in AD patients compared with controls, they could not distinguish AD patients from patients with other neurodegenerative diseases such as vascular dementia and amyotrophic lateral sclerosis (Tumani et al., 1995; Tumani et al., 1999). It may therefore be interesting for future studies to combine these indicated assays with our newly developed sELISA, which is capable of measuring total GS content in CSF using the AFBN protocol, to determine the ratio of decameric versus total GS protein for potential additional discriminatory value.

Although the AFBN protocol improved the detection of MBP and GS, no significant improvement was detected for the measurement of GFAP. This was unexpected as GFAP is often present in multimeric forms (Shaw and Hawkins, 1992) and may be expected to show improved detection upon AFBN treatment as observed for GS. However, the failure of the AFBN treatment to improve the detection of GFAP may indicate that a conformational change in the epitope resulting from AFBN treatment may have occurred that was detrimental to binding of the antibodies used in our sELISA or that the antibodies bind more optimally to the native form of the protein in the untreated CSF. Alternatively, the acidification of GFAP may not be irreversible and the multimers may reform rapidly enough to prevent antibody binding and thus prevent improved detection, or acidification is insufficient to reduce the GFAP to monomers.

AFBN slightly diminished the measurement of Aβ42, t-tau and p-tau in CSF whereas others have shown that the measurement of Aβ42 can be enhanced by the pretreatment of CSF samples with detergent or heat, presumably by dissociation of Aβ oligomers to liberate hidden or masked epitopes (Bibl et al., 2004; Bjerke et al., 2010). We anticipated that the AFBN may improve the detection of Aβ42 either due to release of oligomeric or aggregated forms into smaller forms of the protein or by the release of amyloid β autoantibodies (Li et al., 2007). However, this was not observed in our experiments. The AFBN treatment seems to be insufficient to resolve oligomeric or fibrillar forms of Aβ into smaller oligomeric or monomeric forms, or may even have stimulated its aggregation. It is also possible that, after initial resolution of Aβ aggregates, the subsequent neutralisation of CSF may have permitted the re-aggregation of Aβ42. Although acid treatment of CSF has been shown to improve the detection of autoantibodies to amyloid β (Li et al., 2007), any release of autoantibodies that may have occurred during our AFBN protocol did not lead to improved detection of Aβ42. Finally, it is also possible that the oligomeric or fibrillar fraction of total Aβ42 is simply too small to confer improved detection following AFBN treatment, or that this is already achieved by the buffers supplied with the kits.
When left at room temperature, the pH of CSF quickly rises after withdrawal as a function of time until a peak is reached and it is also affected by freeze/thaw cycles (Bjerke et al., 2010). Furthermore, the efficiency of an antibody to bind to solid phases, such as a microtitre plate, is influenced by the pH of the coating buffer independent of buffer concentration or formulation (Goldblatt et al., 1993). It is suggested that the optimal pH for antibody and antigen binding lies somewhere between the isoelectric point (pI) of the antibody and the antigen (Boenisch, 2006). Hence, the pH of the environment may worsen antigen binding if the pH of the buffer system varies too dramatically from the pI of the antibody. Thus, the efficiency of antibody–antigen binding in ELISAs might be diminished by a high CSF pH. Hence, it could be expected that antigen–antibody binding will be enhanced by neutralisation of the antigen prior to addition to the microtitre plate. Contrary to expectations, this was not observed in any of the homemade or kit ELISAs that we tested.

It can be assumed that the reagents supplied with the kits for Aβ42, t-tau and p-tau have been pre-optimised. Indeed we did not see any improvement in the detection of these proteins with the addition of either a pre-neutralisation step or the AFBN protocol. Rather, we observed a slight worsening of protein detection in some cases.

**Conclusion**

In summary, neutralisation alone of CSF prior to protein measurement was not effective in improving the detection of any of the proteins tested. However, we have demonstrated the utility of an AFBN pre-treatment of CSF for the optimised detection of both MBP and GS suggesting that the denaturing (acidification) step is required to enhance the detection of these proteins by altering protein and/or epitope conformation. Further investigation is required to determine whether the improved detection of these proteins will also enhance their sensitivity and specificity in detecting specific neurodegenerative diseases as suggested for the detection of GDNF (Okragly and Haak-Frendscho, 1997) and cyclic guanosine 5’-monophosphate (cGMP) which have been investigated for use as biomarkers in the diagnosis of amyotrophic lateral sclerosis (ALS) (Grundström et al., 2000; Iłżecka, 2004).

Although we showed that AFBN pre-treatment may not be useful for enhancing the detection of all CSF proteins, it may be a useful protocol to evaluate the possibility of epitope-masking in new biomarker development where similar types of confounders may be expected.

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Glutamine synthetase ELISA
References


Chapter 8

Elevated CSF glutamine synthetase in NMO and multiple sclerosis


Manuscript in preparation

“Oh, I'm not particular as to size,” Alice hastily replied: “only one doesn't like changing so often, you know.”
Abstract

Objective: Neuromyelitis optica (NMO) is often misdiagnosed as multiple sclerosis (MS) due to similarities in the initial neurologic presentation. Some treatments used for MS patients may exacerbate NMO and thus biomarkers to clearly distinguish between NMO and MS are important. NMO patients with a negative result on screening for aquaporin-4 (AQP4) antibodies are difficult to define clinically. Since NMO pathology is primarily astrocyte-mediated, astrocyte secretory proteins may serve as useful biomarkers for NMO. The aim of this study was to determine whether secretion of the astrocytic enzyme glutamine synthetase (GS) is altered in NMO patients (both seropositive and seronegative) compared with controls, MS patients and patients with optic neuritis (ON) in the absence of MS or NMO.

Methods: Using a novel enzyme-linked immunoassay, we measured levels of GS in the cerebrospinal fluid (CSF) of 68 patients with MS, 44 with NMO, 6 with ON, and 37 controls.

Results: Levels of GS were significantly increased in the CSF of NMO and MS patients compared with controls and ON patients.

Conclusions: Levels of GS are significantly increased in NMO and MS but do not distinguish NMO from MS regardless of AQP4 serological status.
Introduction

Inflammatory, demyelinating disorders of the central nervous system such as neuromyelitis optica (NMO) and, in particular, multiple sclerosis (MS) are the major causes of non-traumatic disability in young adults. In early stages of disease, NMO is often misdiagnosed as MS as a result of similar initial neurologic presentation. However, misdiagnosis has significant implications for management of patients since some MS treatments (such as interferon beta) are contraindicated in NMO.

Both MS and NMO are believed to involve an immunologic component but the exact underlying mechanisms causing MS and NMO are not understood. The circulating IgG antibody (NMO-IgG) against aquaporin 4 (AQP4) in the serum and cerebrospinal fluid (CSF) of NMO patients has been identified as a useful biomarker for differentiating NMO from optic neuritis (ON) and MS. This antibody is rarely present in ON (without a diagnosis of MS or NMO) and is absent in MS patients and controls. Although the presence of the antibody in most NMO patients indicates that NMO is distinct from MS, 10-25% of patients diagnosed with NMO are seronegative for this antibody even with the most sensitive assays. Therefore identifying additional biomarkers to distinguish NMO, especially seronegative NMO, from MS would be of significant benefit. Since anti-AQP4 antibodies are directed against receptors in the astrocytic endfeet, this suggests that NMO pathology is primarily astrocyte-mediated. Thus other astrocyte-specific biomarkers may be useful in the detection of NMO, even in the absence of AQP4-Abs.

Recent evidence suggests that glutamate excitotoxicity has a role in the pathophysiology of both MS and NMO. Glutamine synthetase (GS), a primarily astrocytic enzyme, has an important role in glutamate regulation by catalysing the conversion of glutamate and ammonia to non-toxic glutamine. In NMO, evidence suggests that astrocytes are the primary target of disease and that demyelination is secondary to oligodendrocyte death arising from loss of trophic support from astrocytes. Thus, we hypothesised that destruction of astrocytes may cause release of GS into the extracellular space subsequently leading to increased concentrations of GS in the CSF of NMO patients. The aim of this study was to examine the potential for CSF GS levels to distinguish NMO patients from patients with MS, ON (in the absence of MS or NMO), and controls.

Methods

Patients

This retrospective study included 44 NMO and 68 MS patients from 5 centres in Nijmegen (the Netherlands), Lyon (France), San Paolo (Brazil), Barcelona (Spain) and Ulm (Germany). From our databases in Nijmegen we retrieved data from the medical files of 281 patients who attended the MS Centre Nijmegen and who had undergone lumbar puncture (LP) as part of the diagnostic procedure between 1 January 2005 and 11 June 2008. Diagnosis at the time of LP was assessed according to previously described criteria. MS Patients for whom CSF was available were selected from our CSF database in Nijmegen for inclusion in the study (n=60). Among these patients we studied 37 patients with remitting relapsing MS (RR-MS), 15 with secondary progressive MS (SP-MS), and 8 patients with primary progressive MS (PP-MS). We also included CSF from an additional 8 MS patients from Brazil, for whom diagnosis was established using the same criteria as described above, and 6 ON patients from Nijmegen (n=4) and Sao Paolo (n=2) with inclusion diagnosis as...
previously described\textsuperscript{17}.

Our NMO spectrum disorder (NMOSD) group consisted of 29 seropositive and 15 seronegative patients. The inclusion criteria for NMO (which includes NMOSD for the purposes of this paper) were as published by Wingerchuk et al (2007)\textsuperscript{2}. The age-matched non-neurological controls ($n=37$) were selected from a separate database of patients referred to our Neurology Department (Radboud UMC) during the period 2001 to 2009, who underwent LP as part of the diagnostic process, and who were confirmed as having no neurological disease. For these patients routine CSF parameters such as cell count, protein and glucose, were assessed as normal.

\textit{Standard protocol approvals, registrations, and patient consents}

CSF samples were collected according to standard protocols with local ethics approval. At the time of collection all patients from the Radboud UMC gave informed consent to lumbar punctures, including later use for scientific purposes but written consent from the patients was legally not required for our analyses. Informed written consent for lumbar puncture was obtained from all patients from the other participating centres.

\textit{CSF samples}

CSF samples were collected in polystyrene or polypropylene tubes, centrifuged (5 minutes, 860 $g$ at room temperature), and stored at -80 °C. For storage purposes, 20 MS samples had been moved to storage at -20°C, but not more than 6 months prior to analysis. Patient information was decoded to maintain confidentiality. Aliquots of CSF from 7 French NMO patients were received from London on ice but thawed. Although this may have affected the results, we have previously observed that CSF levels of GS are unchanged following at least 3 freeze-thaw cycles (unpublished data).

\textit{GS ELISA}

GS levels in CSF were measured using our previously published home-made sandwich ELISA incorporating an acidification and neutralisation step for enhanced detection\textsuperscript{18}. However, for the current study we used a smaller sample volume (75μl) for standards, blanks, controls, and patient samples.

\textit{NMO-IgG antibody assay}

The serostatus for NMO-IgG was determined using recently described methods\textsuperscript{6, 19, 20}. NMO serostatus was confirmed in the French patients by a more recent cell-based assay for AQP4-IgG\textsuperscript{21} and for the Brazilian cohort by a second cell-based assay method\textsuperscript{22}.

\textit{Statistical analysis}

Levels of GS showed a non-Gaussian distribution for controls and were log-transformed. Between-groups analysis was performed on transformed data using ANOVA and, when controlling for potential confounding factors (age and gender), ANCOVA. Data were analysed using GraphPad Prism, version 5 and SPSS Statistical software, Version 20.
**Results**

Patient demographics and GS levels are provided in Table 1. Age (p=0.49) and gender (p=0.09) did not differ significantly across the disease groups although the difference in age between the MS and control groups approached significance (p=0.06; Table 1). GS levels were not influenced by storage conditions (-20⁰C versus -80⁰C; data not shown). GS levels were associated with age only in the MS group (p=0.02), and tended to be correlated with gender in the control group (p=0.06).

Using ANOVA, we found significantly higher levels of CSF GS for both NMO (p<0.001) and MS (p<0.001) compared with controls (Figure 1) and this was maintained after controlling for age and gender using ANCOVA (p<0.001 for both groups, Table 1). GS did not differ significantly between MS and NMO using ANOVA (p=0.17) but, after controlling for age and gender effects using ANCOVA, we found a small, significant increase in NMO compared with MS (p<0.05). When we further separated the NMO patients into seronegative and seropositive, we found that this effect was true for MS compared with the NMO seropositive patients (p<0.05) but not compared to the seronegative patients (p=0.36). Differences between NMO seropositive and seronegative patients also did not reach significance (p=0.36). CSF GS levels in the ON patients did not differ significantly from controls (p=0.80). Compared with ON, NMO tended to have higher CSF GS levels (p=0.05) and this effect became significant after controlling for age and gender (p<0.01). In this case the effect was observed for both NMO seropositive (p<0.01) and seronegative patients (p<0.05). Levels of total protein in CSF were slightly elevated in the NMO group compared with all other groups (Table 1) but this difference was not significant even after controlling for age and gender.

**Table 1.** Patient demographics and CSF parameters.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MS</th>
<th>NMO</th>
<th>ON</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>37</td>
<td>68</td>
<td>43</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age; yrs (s.d.)</td>
<td>43.2 (11.1)</td>
<td>42.4 (10.7)</td>
<td>45.1 (17.4)</td>
<td>37.4 (10.6)</td>
<td>ns⁺</td>
</tr>
<tr>
<td>Gender; M/F (%male)</td>
<td>18/19 (49%)</td>
<td>17/51 (25%)</td>
<td>12/31 (28%)</td>
<td>2/4 (33%)</td>
<td>ns⁺</td>
</tr>
<tr>
<td>GS (μg/L); mean (s.d.)</td>
<td>243 (143)</td>
<td>411 (255)***</td>
<td>500 (250)***</td>
<td>242 (109)</td>
<td>&lt;0.001ᵇ</td>
</tr>
<tr>
<td>Total Protein (μg/mL); mean (s.d.)</td>
<td>0.50 (0.16)</td>
<td>0.47 (0.21)</td>
<td>0.64 (0.69)</td>
<td>0.37 (0.17)</td>
<td>nsᵇ</td>
</tr>
</tbody>
</table>

⁺ p-value calculated using ANOVA; ***p<0.001 compared with controls;ᵇ calculated using ANCOVA controlling for age and gender. GS: Glutamine synthetase; MS: multiple sclerosis; NMO: neuromyelitis optica; ON: optic neuritis; s.d.: standard deviation; ns: not significant
Discussion

In this study we compared levels of GS in the CSF of MS, NMO, ON and control patients. Degradation of astrocytes is a key pathophysiological feature of NMO and we hypothesised that GS released from damaged astrocytes may be reflected as elevated GS in the CSF of NMO patients compared with controls and MS patients. Indeed we observed increased levels of GS in NMO compared with controls with the greatest differences seen for seropositive patients. This could indicate that GS is released into the CSF as a result of astrocyte damage, particularly in the seropositive NMO patients and could suggest a relationship between GS and the presence of NMO-IgG antibody.

However, the increase in GS levels we observed in NMO is far less striking than expected given the more pronounced increases observed for other astrocytic markers such as glial fibrillary acidic protein (GFAP) and S100b 9, 15, 23-25. Although CSF GS levels were more prominent in NMO seropositive than NMO seronegative patients compared with the MS patients, we did not find significant differences between the seronegative and seropositive NMO patients. Moreover, levels of CSF GS were also significantly higher in the MS patients than controls and these increases in GS levels and could not be used to distinguish between MS and NMO patients.

The observed elevation in GS levels in the CSF of MS patients was intriguing and it is uncertain why GS should be increased in these patients. In the brain, GS is a primarily astrocytic enzyme but there are several studies showing expression of GS in oligodendrocytes 10, 13, 26, 27. Oligodendrocytes are severely damaged in MS and GS immunoreactivity is reduced in both active and silent MS brain lesions compared with unaffected and control tissues 26. Hence release of GS due to oligodendrocyte damage could potentially account for some of the increase in CSF GS levels observed in our MS group. Hypomyelination in brain white matter is associated with a reduction in GS activity caused by metal toxicity as was shown in a recent animal study 28. Thus loss of GS activity could further exacerbate oligodendrocyte damage leading to demyelination or reduced remyelination 29. This notion is supported by studies showing that GS is highly susceptible to oxidative modification and may play a role in glutamate excitotoxicity in MS 26, 30, 31. Additionally, GS has been recently shown to be important for regulating Schwann cell differentiation and promoting remyelination following injury 32 and it has also been speculated from animal models that GS activity in oligodendrocytes may play a role in myelin regulation 33.

Figure 1. GS levels in CSF of controls, MS, NMO and ON. Higher levels of GS were observed in NMO and MS patients compared with controls and ON.

*** p<0.001 compared with controls; § seropositive (closed triangles) and seronegative (open triangles) NMO subgroups. GS: glutamine synthetase; MS: multiple sclerosis; NMO: neuromyelitis optica; ON: optic neuritis.
The increased levels of GS in the CSF of MS and NMO patients could also potentially be explained by leakage of GS from blood into the CSF since GS is also highly expressed in kidney, liver, skeletal muscle, spleen, and heart. Although, it has been estimated that less than 2% of GS in the CSF arises from non-intrathecal origin, leakage of GS from blood into CSF in patients with a compromised blood-CSF barrier, is highly likely. Both MS and NMO have been associated with compromised blood-CSF barrier dysfunction, particularly in NMO patients, where CSF total protein levels are frequently elevated. This may potentially have contributed to the higher levels of GS seen in the NMO patients compared with the MS patients. Although CSF total protein was higher in the NMO group (particularly the NMO-seropositive patients) than all other groups (Table 1), the values were highly variable and no significant between-group differences in CSF total protein levels were observed to support this hypothesis.

A major strength of our study is the inclusion of a large well-defined group of NMO patients from a number of different institutions. However, we acknowledge that the retrospective nature of our study may somewhat limit our ability to generalise our findings to other patient populations, particularly since the time of lumbar puncture with respect to clinical symptoms was not known for most patients. This may have implications for our findings given that others have shown that levels of other CSF proteins can differ between relapse and remission. Finally, previous studies have shown that NMO-IgG is absent in MS patients and controls and rarely present in ON but NMO-IgG analysis was neither analysed in the majority of our MS and ON patients nor in controls. Therefore, we cannot entirely exclude that anti-AQP4-positive, non-NMO patients may have been included in these groups, thereby influencing the interpretation of our results.

In summary, we showed that CSF GS concentrations are increased in MS and NMO compared with controls. However, a larger prospective study with the inclusion of a broader range of diseases with large patient numbers will be required to determine the extent to which GS is altered in demyelinating diseases.
References
Glutamine synthetase levels in neuromyelitis optica and multiple sclerosis

Part 5

Concluding Remarks

Alice thought to herself "I don't see how he can ever finish, if he doesn't begin."
Chapter 9

General discussion and future perspectives

“No, no! The adventures first, explanations take such a dreadful time.”
General discussion and future perspectives

Neurodegenerative diseases are chronic and frequently debilitating disorders of the nervous system that usually have an insidious onset. A major challenge in diagnosing neurodegenerative diseases arises from symptom profiles that exhibit not only high variability among patients with the same disease, but which frequently mimic other neurodegenerative disorders with similar symptoms. Another major problem in the diagnostic process is the early identification of disease. There is increasing evidence that the pathological processes underlying neurodegenerative diseases may begin to develop many years prior to the onset of overt symptoms 1-3. This so-called ‘pre-clinical stage’ of disease, in which there is an absence of clinical signs and symptoms, can occur in the face of extensive underlying pathological damage 4. Therefore, for many patients, disease diagnosis remains uncertain for extended time periods (up to years). Definitive confirmation of the diagnosis is often only possible during post mortem examination of the brain to reveal the distinguishing hallmark pathological features characteristic of an individual neurodegenerative disease. Since it is not possible to easily obtain brain biopsy samples during the life of the patient for confirmation of disease, biomarkers from other appropriate sources are needed. In this thesis we focused on the assessment of potential cerebrospinal fluid (CSF) biomarkers for their use in the differential diagnosis of selected neurodegenerative diseases.

**Biomarkers of disease**

Biomarkers have been defined as “…cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells or fluids” (Hulka et al., 1990, cited in 5). They can involve direct measurement of certain components of body fluid (e.g. blood, urine, CSF) or indirect measurements such as brain imaging (e.g. MRI, computed tomography scan), which estimate changes in composition or function of the relevant components. Based on the close anatomical relationship between the brain and CSF it has been postulated that the composition of the CSF might reflect early ongoing disease processes occurring in the brain 6-8. Thus, we hypothesised that some well-chosen CSF constituents might represent diagnostic biomarkers for neurodegenerative diseases and their differential diagnosis. Although CSF collection requires a procedure that involves minor risks it has the advantage over blood or urine of being less susceptible to contamination arising from systemic infections and biological degradation in the liver or kidney 5, 9, 10. Therefore, CSF represents an important medium for the identification and development of biomarkers of neurodegenerative disease.

**CSF biomarkers for differential diagnosis**

**Dementia disorders**

In recent years there have been great advances in the development and evaluation of CSF biomarkers for the diagnosis of Alzheimer’s dementia. The three most rigorously investigated biomarkers for Alzheimer’s disease - amyloid beta (Aβ1-42), tau and phosphorylated tau proteins – are increasingly being considered for diagnostic purposes in clinical practice 11-14. While none of these biomarkers show sufficiently high utility as a stand-alone biomarker for Alzheimer’s disease, combinations of these biomarkers have been shown to have high sensitivity and specificity for separating Alzheimer’s disease from non-demented patients and healthy controls, providing up to 92% sensitivity for the
detection of Alzheimer’s disease and up to 89% specificity for Alzheimer’s disease 14, 15. Despite the utility of these biomarkers in detecting and predicting Alzheimer’s disease based on the underlying neural basis of the disease, they have poorer clinical utility for discriminating Alzheimer’s disease from other forms of dementia 8, 16-18. The primary differential diagnoses for Alzheimer’s disease include dementia with Lewy bodies, vascular dementia and frontotemporal dementia 19. Difficulty distinguishing between these dementia forms has significant clinical implications because the medications used in the treatment of one dementia disorder can be contraindicated in another dementia disorder. For example, neuroleptics used for the treatment of behavioural disorders in Alzheimer’s disease can lead to worsening of symptoms, or even death, in patients with dementia with Lewy bodies who often show a pathological sensitivity to these medications 20, 21.

A second lesson these studies teach us is that an individual biomarker meeting all the criteria required of a stand-alone biomarker may not be feasible, and that combinations of biomarkers may be necessary to provide optimal diagnosis and differential diagnosis of dementia disorders 1, 16, 22, 23. In keeping with this notion, in Chapter 2, we examined the utility of adding 3-methoxy-4-hydroxyphenylglycol (MHPG), a metabolite of the neurotransmitter noradrenaline, to the traditional CSF biomarkers for Alzheimer’s disease to differentiate dementia with Lewy bodies from Alzheimer’s disease and other forms of dementia. Our choice of MHPG, was based on observations in other investigations 24-26 showing that metabolism of noradrenaline is altered in patients with dementia with Lewy bodies, a disease that shares many similar clinical features of Alzheimer’s disease. In a previous study 24, we showed that using MHPG in combination with Aβ1-42 and tau proteins improved the ability to distinguish DLB from Alzheimer’s disease above the traditional combination of biomarkers alone 24. Furthermore, the results indicated an altered noradrenaline metabolism in dementia with Lewy bodies that was different from that of Alzheimer’s disease. A review of the literature suggested that this might also be the case for frontotemporal dementia and vascular dementia 26-30. Thus, in Chapter 2, we examined whether this same combination of biomarkers might also be useful in discriminating dementia with Lewy bodies from other forms of dementia. In the study we found that the addition of MHPG to the signature biomarkers improved the sensitivity for correctly detecting dementia with Lewy bodies in our study participants from 62% to 65% and improved our ability to distinguish Alzheimer’s disease from dementia with Lewy bodies from 92% to 100% specificity among our study participants. Thus we confirmed our previous findings that MHPG combined with traditional biomarkers could improve the distinction between Alzheimer’s disease and dementia with Lewy bodies, which supports the idea that combinations of biomarkers may be required for optimal diagnosis of disease. However, we were unable to show that this combination of biomarkers could aid in the differential diagnosis of dementia with Lewy bodies from frontotemporal dementia or vascular dementia, indicating that additional yet-to-be-identified biomarkers are required for this purpose.

A further weakness of the traditional combination of Aβ1-42, tau and phosphorylated tau in the diagnosis of Alzheimer’s disease is in that detection of Alzheimer’s disease in early stages of disease remains difficult. Although combinations of biomarkers can help to predict the progression of cognitively normal individuals or those with mild cognitive impairment to Alzheimer’s disease 1, 31, 32, additional biomarkers would be useful. The peptidyl-prolyl isomerase, Pin1, has been shown to be involved in the regulation of both
Aβ1-42 and tau, and loss of activity of Pin1 during conditions of oxidative stress may be involved in the pathophysiology of Alzheimer’s disease. Therefore, we aimed to examine the potential of Pin1 as an alternative diagnostic marker for Alzheimer’s disease that might also identify Alzheimer’s disease in its early stages (Chapter 3). We were able to develop and apply an immunoassay to detect oxidised Pin1 in brain tissues and found that levels of oxidised Pin1 were increase by about 9%, and the ratio of oxidised Pin1 to Pin1 by approximately 20%, in early stages of disease compared with controls, which is consistent with previous studies. However, we were not able to determine the value of (oxidised) Pin1 in CSF as a biomarker for the differential diagnosis of dementia due to undetectable levels in the CSF. Therefore, additional research will be required to develop more sensitive measures of detection to determine whether levels of oxidised Pin1 might differ between the various forms of dementia.

Movement disorders

In case of movement disorders, the major differential diagnoses for Parkinson’s disease are other conditions associated with a hypokinetic-rigid syndrome including the atypical parkinsonisms: multiple system atrophy, progressive supranuclear palsy and corticobasal syndrome. Several reports have now shown that CSF levels of neurofilament protein can differentiate PD and controls from patients with atypical parkinsonisms but can neither differentiate between controls and Parkinson’s disease nor between the different forms of atypical parkinsonism. The results presented in Chapter 4 have confirmed these findings in an independent study strengthened by the inclusion of both discovery and validation cohorts. Using NFL alone we were able to detect the presence of MSA with a sensitivity of 74% and could distinguish MSA from PD with 92% specificity in our patient cohort. Together these findings support the use of NFL as an adjunct to clinical practice for improving the diagnostic accuracy through enhanced discrimination of Parkinson’s disease from atypical parkinsonisms. However, although NFL clearly distinguishes atypical parkinsonisms from Parkinson’s disease, its inability to be used as a specific biomarker for a single disease can be viewed as a major short-coming of this biomarker. In particular, its inability to distinguish Parkinson’s disease from control subjects highlighted the need for additional disease-specific biomarkers.

For the purpose of identifying disease-specific markers for Parkinson’s disease we attempted, in Chapters 4 and 5, to verify previous findings that fms-like tyrosine kinase receptor ligand (FLT3L), a recently identified novel biomarker, and DJ-1, a moderately well-studied biomarker, could be used to distinguish Parkinson’s disease from controls and multiple system atrophy. One previous study using western blot experiments showed increased levels of DJ-1 in Parkinson’s disease compared to controls and this finding was supported by our own findings using ELISA assays (Chapter 5). In this study we found an increase of about 26% in Parkinson’s disease above that of controls that allowed us to detect Parkinson’s disease with rather high accuracy (81%) but more moderate specificity for Parkinson’s disease (52%). Interestingly, we found for the first time that levels of DJ-1 were 70% higher in multiple system atrophy than in controls and 35% higher than those with Parkinson’s disease, thus enabling a clear distinction between controls and multiple system atrophy (sensitivity of 78% and specificity of 100%), and, to a lesser extent, between multiple system atrophy and Parkinson’s disease (sensitivity of 78% and specificity of 78%). In contrast to the DJ-1 findings, we were unable to confirm a previous finding that CSF levels of FLT3L could distinguish between these diseases (chapter 4). While DJ-1 showed
promise for use as a biomarker, our results contradict other studies which show lower levels of CSF DJ-1 in both Parkinson's disease and multiple system atrophy compared with controls, but no differences between Parkinson's disease and multiple system atrophy 41, 42. Our results were even more contradictory compared with yet another study showing significantly lower levels of CSF DJ-1 that distinguished both Parkinson's disease and multiple system atrophy from controls but not from each other 41. Additional research will be required to determine the reason for these discrepancies, which possibly arise from differences in the methodologies used for DJ-1 and FLT3L quantification in CSF.

Alterations in neurotransmission, particularly altered levels of the neurotransmitter dopamine, can have significant impact on the initiation of movement and have significant implications in the (patho)physiology of Parkinson's disease and related disorders. In another parkinsonian disorder, vascular parkinsonism, the clinical symptoms arise due to vascular insults, such as transient ischaemic attacks or cerebrovascular accident (stroke). We hypothesised that the subsequent vascular injury caused by these insults is likely to have a very different effect on neurotransmitter metabolism compared with Parkinson's disease in which degeneration occurs in a very specific subset of neurons. Very few studies have compared differences in neurotransmitter dysfunction in these two disorders. Thus, we investigated whether physiological differences in CSF levels of neurotransmitters could represent possible biomarkers for differentiating between Parkinson's disease and vascular parkinsonism. For this purpose (Chapter 6), we examined differences in CSF levels of neurotransmitter metabolites from three of the most abundant and physiologically important neurotransmitters - 3-methoxy-4-hydroxyphenylglycol (MHPG), a metabolite of the neurotransmitter noradrenaline; homovanillic acid (HVA) a metabolite of dopamine; and 5-hydroxyindolacetic acid (5-HIAA), a metabolite of serotonin. We found no significant differences in MHPG levels between the patient groups but did observe small, significant differences in CSF levels of 5-HIAA and HVA between Parkinson's disease and vascular parkinsonism although these differences were not sufficiently significant to warrant further investigation of these indices as potential biomarkers. However, in the patients with vascular parkinsonism CSF levels of the dopamine metabolite, HVA, were generally elevated compared with controls. This finding may help to partly explain why many patients with vascular parkinsonism generally respond more poorly to the Parkinson's disease medication, levodopa (a dopamine replacement medication) since the normal to slightly elevated CSF HVA levels are not consistent with dopamine deficiency in patients with the vascular parkinsonism in our study. This has important implications for management of patients with vascular parkinsonism and warrants further investigation.

**Demyelinating disorders**

Multiple sclerosis is the most common chronic demyelinating disorder of the central nervous system. However, neuromyelitis optica, a severe inflammatory demyelinating disease principally targeting the optic nerves and spinal cord, is often difficult to distinguish from multiple sclerosis, particularly when these diseases are in their early stages. Recently, autoantibodies against the water-channel receptor aquaporin 4 (AQP4) were found in large numbers of patients with neuromyelitis optica 43, 44. These AQP4 receptors are associated with astrocytic end-feet and damage to the receptors was found to be associated with the release of high levels of glial fibrillary acidic protein (GFAP) and S100b from astrocytes into the CSF 45, 46. While AQP4 antibodies are not found in all patients with neuromyelitis optica, markedly increased CSF levels of GFAP and S100b are
frequently observed in patients with neuromyelitis optica patients, suggesting that these parameters may be more specific for neuromyelitis optica but they do not distinguish seropositive from seronegative neuromyelitis optica patients. It remains unknown whether seronegative neuromyelitis optica patients represent a separate disease entity, with different underlying pathology or aetiology than seropositive patients, or whether the levels of autoantibodies are, as yet, too low to be detected in these patients. We hypothesised that other astrocytic proteins might also be elevated in neuromyelitis optica and we were curious to see whether levels of glutamine synthetase may differ between seronegative and seropositive patients, thus giving possible clues to pathological differences between these two groups.

Glutamine synthetase, an enzyme involved in the conversion of glutamate and ammonia to glutamine, was chosen as it is generally considered to be an astrocyte-specific enzyme in the brain. We developed an assay for the detection of glutamine synthetase in CSF (Chapter 7) and used this assay to measure levels of glutamine synthetase in the CSF of patients with neuromyelitis optica compared with multiple sclerosis and controls (Chapter 8). While we were able to show that glutamine synthetase levels in patients with neuromyelitis optica were double that of the controls and approximately 22% greater in neuromyelitis compared with multiple sclerosis after controlling for confounding factors, we found high heterogeneity in the levels measured, particularly for the neuromyelitis and multiple sclerosis groups. Furthermore, there were no significant differences in glutamine synthetase levels between seronegative and seropositive neuromyelitis optica. Therefore, glutamine synthetase was not useful as a biomarker for neuromyelitis optica. Furthermore, multiple sclerosis patients also had significantly higher levels of glutamine synthetase than the control group, again with considerable overlap between multiple sclerosis and controls.

Although not useful as a biomarker for either neuromyelitis optica or multiple sclerosis, the elevated levels of glutamine synthetase may be able to provide some insight into possible underlying mechanisms of pathology of multiple sclerosis and/or neuromyelitis optica. There is some debate in the literature regarding the specificity of glutamine synthetase as a purely astrocytic enzyme, with several papers indicating significant levels of glutamine synthetase in myelin-producing oligodendrocytes. Thus the high levels of CSF glutamine synthetase measured in our multiple sclerosis patients may be indicative of damage to oligodendrocytes and subsequent release of glutamine synthetase into the CSF. However, additional studies will be required to confirm or refute this hypothesis.

**CSF biomarkers – what are the pitfalls?**

**CSF biomarkers – still missing the diagnostic mark**

To date the most well studied biomarkers for any neurodegenerative disease are the signature biomarkers of Alzheimer’s disease, Aβ1-42, tau and phosphorylated tau. These biomarkers have been shown to correlate highly with AD-associated pathology – senile plaques and neurofibrillary tangles – and may have potential as early markers of disease. On the other hand, they do not correlate well with the severity of cognitive dysfunction which would give an indication of disease progression. The poor correlation of signature biomarkers with the clinical symptoms shows that Alzheimer’s disease-type pathology can occur in the absence of clinical symptoms and, conversely, significant clinical signs and symptoms may occur in patients with less extensive brain pathology, possibly
indicating a different disease aetiology 4, 47. In other cases, changes in brain pathology have been shown to occur in patients who later go on to develop Alzheimer’s disease 48. Thus, while the signature biomarkers of Alzheimer’s disease seem to be good at detecting Alzheimer-type brain pathology, they miss the mark in terms of their correlation with clinical symptoms, showing more accuracy in ruling the disease out rather than confirming the presence of clinical disease. That is, if the signature biomarkers do not indicate the presence of Alzheimer-type pathology, then the patient is unlikely to have – or develop in near future - dementia of the Alzheimer type. On the other hand, when biomarker levels suggest the presence of Alzheimer-type brain pathology, this will not necessarily indicate the presence of clinical Alzheimer’s disease. Since optimal biomarkers can be used not only to validate the presence or absence of disease but can also be used for monitoring disease progression and treatment effects, the above-mentioned signature biomarkers seem to be not more than poor predictors.

Disease diversity – the problem of mixed pathology

Disease diversity adds an additional complexity to our search for biomarkers for differential diagnosis. Firstly, there have been many cases in which patients are found to have mixed brain pathology 49-53. That is, they show signs of having more than one neurodegenerative disease. For example, Alzheimer’s disease can occur together with another form of dementia and, similarly, dementia often occurs in patients with Parkinson’s disease. Secondly, for most neurodegenerative disorders, multiple neurotransmitter systems are involved, but their extent varies from patient to patient, and also depends on the disease stage and on the age at onset of the disease. For example, Parkinson’s disease is typically equated with a dopaminergic lesion, but certainly in more advanced disease stages, additional neurotransmitter systems become affected as well. Thirdly, vascular risk factors such as hypertension, diabetes and hypercholesterolemia not only increase with age but are associated with an increased risk for the development of neurodegenerative disease. Examples include both Parkinson’s disease and Alzheimer disease 54. Moreover, vascular factors often contribute to the severity of neurodegenerative disease symptoms, particularly cognitive impairment or dementia 55-57. This adds a new layer of complexity to clinical symptoms and likely contributed to the heterogeneity in CSF values that we observed in the studies conducted in Chapters 2 and 6 in which we compared disorders with a vascular component (vascular dementia and vascular parkinsonism) with their neurodegenerative counterparts (Alzheimer’s dementia and Parkinson’s disease, respectively).

Vascular damage is highly heterogeneous (variable) and individual patients have unique vascular damage. Although imaging techniques are useful for identifying vascular lesions, vascular damage can occur in 30-60% of patients with Alzheimer’s disease and between 25 to 80% of patients with vascular dementia will have comorbid Alzheimer’s disease 58 and so identifying vascular lesions has little diagnostic value in distinguishing between Alzheimer’s disease and vascular dementia. Imaging studies can be prone to overestimation of the presence of vascular dementia and underestimation of the presence of neurodegenerative co-pathology 59. In this respect, use of the signature biomarkers of Alzheimer’s disease in conjunction with clinical assessment and imaging studies, have been shown to improve the differential diagnosis between vascular dementia and Alzheimer’s disease, and to detect the presence of co-pathology. In other cases, vascular pathology can be so localised (focal) as to cause clinical symptoms that are indistinguishable from its
neurodegenerative counterpart. For example, focal lesions of the basal ganglia can result in symptoms of Parkinson's disease 60, and co-morbid vascular lesions are more common in Parkinson patients compared to the normal population 54. Since vascular damage is unique to individuals and will lead to diverse clinical phenotypes among patients, specific biomarkers may be more feasible for dementias with a neurodegenerative component whereby all patients with the same type of dementia may be expected to have the same deficit (e.g. a protein deficiency).

Biomarkers for diagnosis and differential diagnosis

Ultimately, we aim to use biomarkers to determine the specific disease faced by an individual patient. That is, we aim to determine the patient's 'diagnosis'. Diagnosis is based on the identification of the patient's signs (objective measures) and symptoms (subjective measures) and their fit with internationally established criteria for a particular disease entity. However, an important part of diagnosis is 'differential diagnosis', which is the process of identifying all possible diagnoses and then ruling out individual diseases until only one possibility remains. This is a particularly necessary part of the diagnostic process when patients present with signs and symptoms that can be attributed to more than one disease. While biomarker research often places emphasis on the role of biomarkers in distinguishing disease from a healthy condition, less emphasis is placed on biomarkers that can be used to distinguish between diseases with similar signs and symptoms. In this respect, neurofilament protein appears to have substantial potential as a biomarker for differential diagnosis since it clearly distinguished Parkinson's disease from all other atypical parkinsonisms studied (Chapter 4). However, it lacked potential as a specific diagnostic marker since it could neither discriminate Parkinson's disease from controls nor distinguish between the atypical parkinsonisms studied. All atypical parkinsonisms were consistently associated with elevated levels of neurofilament protein, whereas Parkinson's disease had levels of neurofilament proteins that were comparable to levels found in both healthy controls and controls with non-neurodegenerative disorders 36, 37, 39, 61. Whereas clinical diagnosis based on the patient's signs and symptoms indicated the presence of Parkinson's disease, control-comparable levels of neurofilament protein excluded the likelihood of an atypical parkinsonism and added weight to a more likely diagnosis of Parkinson's disease.

With respect to Alzheimer's disease, the signature biomarkers have been shown to be diagnostic for Alzheimer's pathology and can distinguish Alzheimer's disease and other dementias from controls. However, biomarkers for differential diagnosis with the specificity and sensitivity required to distinguish between different forms of dementia are still lacking. Alternative combinations of the signature biomarkers, such as the ratio of p-tau/ Aβ1-42 62 have been shown to have some utility in distinguishing Alzheimer's disease from other forms of dementia and, as mentioned previously, we found that the addition of MHPG to the signature biomarkers showed enhanced differentiation of Alzheimer's disease from dementia with Lewy bodies but not from frontotemporal dementia or vascular dementia (Chapter 2).

Unlike the biomarkers tested for use in movement disorders and dementia, autoantibodies against the aquaporin-4 water channel receptor appear to represent a biomarker that is not only specific, and diagnostic, for neuromyelitis optica but also distinguishes neuromyelitis optica from multiple sclerosis. Unfortunately, AQP4 also falls short as a biomarker with regard to its sensitivity in that a subset of patients with neuromyelitis optica is negative
for these autoantibodies. It is uncertain whether the currently available assays simply lack the sensitivity to measure low levels of autoantibodies in these patients, or whether these patients represent a different form of neuromyelitis optica. Our attempt to resolve this issue using measures of glutamine synthetase to distinguish between seropositive and seronegative neuromyelitis patients (Chapter 8) hinted that seronegative and seropositive neuromyelitis optica may represent separate disorders but the differences were much too small to be conclusive. Therefore, these markers are still insufficient for distinguishing seronegative and seropositive neuromyelitis optica from each other and from multiple sclerosis so additional research will be required to resolve this incongruity and supplementary biomarkers will be required to differentiate between the individual disorders, in the case that future research shows that seronegative and seropositive neuromyelitis are indeed separate disorders.

Technical barriers
In addition to the shortfalls in diagnostic performance, researchers are faced with a multitude of technical issues that can arise in biomarker research. Clear evidence of altered brain protein levels in a particular disease condition compared to controls is not always reflected in the levels measured in CSF. Examples can be found in Chapter 3 where we observed that levels of the proteins (Pin1 and oxidised Pin1) were (almost certainly) too low to be measured in the CSF, and in Chapter 7 where we found that epitope masking seemed to prevent the binding of the antibody to the antigen thus requiring acid treatment to unmask the epitope. Therefore, additional (pre-analytical) processing or more sensitive technologies will be required to improve the detection of proteins, particularly those present in very low levels. Secondly, different methods available for the measurement of the same protein often seem to provide different results. This may have explained the differences in results we found using ELISA methods for the measurement of FLT3L (Chapter 4) and DJ-1 (Chapter 5) compared with other papers in which either a different ELISA method or an alternative methods (using Luminex technology) were used for the measurement of the same proteins. Therefore, the implementation of biomarkers for use in clinical practice will require strict evaluation and standardisation of the methods used for detection.

Limitations of the research
The retrospective nature of the studies performed presents a limitation for the work performed in this thesis. Firstly, the Radboud University Medical Centre is a teaching hospital highly focussed not only on high standard health care provision as part of routine healthcare, but also acts as a tertiary referral centre for complex cases. As such, many more referrals are received for the investigation of patients with complex symptomatology, and for whom diagnosis is not clear-cut based on the presenting clinical symptoms, than might be expected in smaller hospital settings. Furthermore, lumbar puncture is generally performed only in cases where diagnosis is uncertain, or as part of dedicated research protocols in selected cases. This likely biased the selection of patients included for the studies particularly given that we only included patients for whom lumbar puncture had been performed as part of the diagnostic procedure. In addition, we excluded patients for whom the diagnosis remained uncertain despite extensive follow-up. Although this study design ensured that we had the most accurate diagnosis possible, this resulted in lower numbers of patients available for inclusion in the studies and reduced our ability to generalise our findings to a wider population. Finally, the lack of neuropathological
confirmation of disease meant that we could not confirm whether our diagnoses based on extensive neurological assessment and protracted follow-up were indeed as accurate as expected from post-mortem examination.

Ultimately, these issues will need to be addressed by additional evaluations of much larger, more general populations to accurately determine the true sensitivity and specificity of these biomarkers and final diagnoses will require additional confirmation with post-mortem neuropathological assessment of the patients. Unfortunately, this will require extensive resources, complex multi-centre institutions and extended periods of time that will require thorough coordination and monitoring that may be impractical.

Future perspectives
Despite the current lack of specific CSF biomarkers for diagnosis and differential diagnosis of neurodegenerative diseases, the outlook for future biomarker discoveries remains optimistic.

Prospective study design
As mentioned in the limitations section, the retrospective nature of the studies performed for this thesis may have introduced biases that could have influenced the analysis of the data. In addition, the CSF data were analysed in isolation, not in conjunction with clinical symptoms, and we were dependent upon clinical records for the most recent and accurate diagnoses. Thus, we were unable to adequately assess the correlation between the assessed biomarkers and clinical symptoms. In order to address these issues, we currently have prospective studies on parkinsonism in progress that have been designed to evaluate and compare the initial diagnosis with the final diagnosis (after 3 years) of each patient and to evaluate the predictive value of ancillary markers (including CSF biomarkers and imaging methods) taken at baseline with the final diagnosis (van Rumund et al, paper in preparation). This will enable us to develop optimal diagnostic models based on baseline characteristics and clinical variables in combination with ancillary investigations where these are assessed as having added value in the diagnostic model. Furthermore, the prospective nature of the study is also predicted to yield additional insights into the disease progression and succession of symptoms that may help us to identify the prognostic value of the CSF biomarkers measured.

The ‘-omics’ to identify potential biomarker targets
Recent research has focussed on the development of ‘-omics’ techniques investigating changes in metabolism (metabolomics), expression profiling (transcriptomics), proteins (proteomics) and genes (genomics) using both targeted and untargeted approaches as a means of biomarker discovery. These techniques generate enormous amounts of data used to identify potential biomarkers of disease and, although approaching biomarker discovery from different perspectives, are often successful in identifying potential disease related protein biomarkers. Causative genetic mutations for familial diseases discovered by genomics has often directed research towards the specific proteins affected by these gene mutations and how these proteins are altered in sporadic forms of disease. One of the first examples of this was the discovery of missense mutations in the alpha-synuclein (SNCA) gene in 1997. In Alzheimer’s disease three highly penetrate genes, amyloid beta precursor protein, PSEN1, and PSEN2, have been shown to account of a small number of familial cases of Alzheimer’s disease which are not shown in non-Mendelian inherited disease. In sporadic cases, persons who inherit apolipoprotein 4 (APOE4)
alleles have an increased risk (3 times for one allele or 10 times if they have inherit alleles from both parents) compared with persons who don't carry APOE4 alleles 74. Although genome wide association studies have found other susceptibility genes associated with non-Mendelian inherited Alzheimer's disease, they confer only a small increase in disease risk and only account for a small part of the genetic risk of Alzheimer's disease 75.

Proteomics is the study of the structure and function of proteins which can be performed using a variety of methods including 2-dimensional gel electrophoresis or combined liquid chromatography and mass spectrometry 76, 77. These approaches together with sophisticated computational software analyses provide an attractive approach for identifying potential novel biomarkers for the diagnosis and differential diagnosis of neurodegenerative diseases 78, 79. These techniques enable the analysis of large amounts of data from single experiments that can be used to identify changes in molecular profiles between patient groups thus identifying novel biomarkers. Using these approaches multiple proteins have been identified as potential biomarker candidates 78. Most of these approaches use a bottom-up approach involving enzyme digestion of the sample followed by mass spectrometry for the identification of resultant peptides from which the protein composition of the samples can be inferred 80. These techniques are now highly developed and provide a convenient tool for identifying potential biomarker candidates. However, technologies available for analyzing whole proteins (top-down proteomics) are increasingly being developed. The advantage of these approaches is that proteins can be examined in their intact state without enzymatic digestion enabling better characterization of the composition and structure of individual protein molecules and likely improves the identification of post-translational modifications that may be present 80.

‘Biomarker panels’ for diagnosis and differential diagnosis of neurodegenerative diseases

In the absence of single biomarkers for the diagnosis of disease, there are indications that a set of biomarkers may more accurately differentiate between individual diseases 17, 36, 81. Indeed different combinations of the signature AD biomarkers have a better diagnostic performance than individual biomarkers 14, 82, 83. As previously discussed, this may come in the form of additional CSF parameters such as we show with the addition of MHPG to the signature Alzheimer disease biomarkers (Chapter 2) which improved the discrimination of Alzheimer's disease from dementia with Lewy bodies 24. Indeed many of the CSF biomarkers tested to date remain sub-optimal, and the development of panels of biomarkers to improve the diagnosis and differential diagnosis of neurodegenerative disease seems viable. While we currently rely on CSF as a source of fluid biomarkers, future combinations of other non-signature biomarkers 84, 85 in various body fluids may also play an important role in the differential diagnosis of neurodegenerative disorders such as miRNAs in blood 86. Furthermore, the use of fluid biomarkers in combination with advanced imaging techniques such as positron emission tomography, magnetic resonance imaging may also help to resolve some of the difficulties with differential diagnosis 87-89.
Assays for measurement of modified proteins

A different approach to identifying potential biomarkers involves the analysis of alterations in protein structure, conformation or activity. Many of the proteins identified as potential biomarkers for neurodegenerative diseases have been shown to be modified in individual diseases compared with controls. These can include changes in conformation such as occurs with unfolding or misfolding of proteins 90-92, increases in the oligomeric forms of the protein compared with monomeric forms 93-95, or structural changes occurring due to post-translational modifications of the protein such as oxidation, glycation or phosphorylation 33, 35, 96, 97. Hence new methods will have to be developed to detect these altered proteins, such as the ELISA we designed to measure the oxidised form of Pin1 (Chapter 3). Other ELISAs have been developed for the measurement of protein isoforms (e.g. Aβ 38, 40 and 42) 98, 99, phosphorylated proteins 96, 100 and oligomeric forms of proteins 101, 102 and some of these methods may be promising for future use as biomarkers.

Development of more sensitive assay methodologies

There are continual developments in technologies to improve the detection and quantification of proteins even at extremely low concentrations in the CSF. Some of the techniques that have shown promising advances include: Mass-spectrometry approaches or SRM-MS combined with top-down proteomics 103-108; nanoparticle technology platforms 109, 110; magnetic and gold particle bead technologies 86, 110. Ongoing development of these techniques will further improve the range of proteins that can be studied and may improve the sensitivity of available biomarkers.

Conclusions

There is a real need for reliable biomarkers that can be used to assist in the differential diagnosis of neurodegenerative diseases, preferably in early clinical or even preclinical stages. In this thesis we explored the biomarker potential of several CSF proteins and metabolites, and we assessed this for three groups of neurodegenerative disorders: dementias; the various forms of parkinsonism; and demyelinating diseases. Some of these CSF biomarkers showed potential to become useful as adjuncts to clinical practice to improve the differential diagnosis of neurodegenerative disorders. However, individual biomarkers for specific diagnosis seem to be elusive. In their absence, panels of fluid biomarkers, in conjunction with clinical assessment and neuroimaging, may provide suitable substitutes. On the optimistic front, there remains hope that emerging candidate biomarkers used alone or in conjunction with other biomarkers, may provide ideal diagnostic tools in the future so that affected patients and their relatives will be able to name their disease rather than facing an uncertain diagnosis.
References


General discussion and future perspectives

“Speak English!’ said the Eaglet. ‘I don’t know the meaning of half those long words, and I don’t believe you do either!”
Summary in English

As discussed in Chapter 1 (Part 1 of the thesis), establishing biomarkers for the differential diagnosis of neurodegenerative diseases is of paramount importance for ensuring optimal medical management of patients. Currently, distinguishing one neurodegenerative disease from another can be rather difficult when using clinical symptoms alone, certainly in early disease stages when the clinical picture is often still incomplete. Moreover, in the early phases of disease, many neurodegenerative diseases share common symptoms, the rate of disease progression is not yet apparent, and the response to medical treatment is often not yet fully clear. Therefore, additional tools to help clinicians in the differential diagnosis of neurodegenerative diseases are needed. The primary aim of this thesis was to examine the diagnostic potential of a selection of CSF proteins that were identified from the literature as biomarkers for some of the most common neurodegenerative diseases. Moreover, we looked at the utility of these biomarkers in distinguishing one neurodegenerative disease from another with similar symptomatology at the time of CSF acquisition. In this thesis the use of CSF biomarkers for differential diagnosis of dementia (Part 2), the differential diagnosis of movement disorders (Part 3), and the differential diagnosis of demyelinating disorders (Part 4).

In order to develop a biomarker, it first needs to be measurable. One of the obstacles faced by researchers looking for biomarkers in cerebrospinal fluid is the difficulty faced in detecting individual proteins in complex biological matrices like cerebrospinal fluid. There may be interactions between proteins, or post-translational modifications of proteins that can hamper protein detection, or target proteins may exist in such low levels in the biological samples that the assay being used is insufficiently sensitive to detect the protein at such low levels. Hence, in Chapter 7 we investigated a technique to enhance the detection and quantification of a selection of relevant proteins using a pre-treatment protocol designed to enhance the detection of proteins in enzyme-linked immunosorbent assay based (ELISA) methods. Indeed, we were able to improve the measurement of some of the proteins tested by applying a technique which appeared to act by exposing additional antigen epitopes, thus enhancing antibody binding and subsequent detection. This technique seemed to be particularly useful for larger proteins, particular those normally present in oligomeric forms.

Part 2 of the thesis focussed on improving the differential diagnosis of dementias. We first investigated how we could build upon the already well-established biomarkers (Aβ, tau and phosphorylated tau) for Alzheimer’s disease by adding an extra measure. In a previous study we had determined that the addition of 3-methoxy-4-hydroxyphenylglycol (MHPG; a metabolite of the neurotransmitter norepinephrine) to the traditional Alzheimer biomarkers (Aβ, tau and phosphorylated tau) could aid in the differentiation of Alzheimer’s disease from dementia with Lewy bodies. In Chapter 2, we examined whether we could confirm these results in independent groups of patients and we addressed the question of whether this combination of biomarkers could also improve the differentiation of dementia with Lewy bodies from frontotemporal dementia and vascular dementia. Indeed we showed that the addition of MHPG to the signature biomarkers improved the sensitivity for correctly detecting the disease in our study participants from 62% to 65% and also improved the ability to differentiate between Alzheimer’s disease and dementia with Lewy bodies. This confirmed our previous results, showing that the addition of MHPG to the traditional combination of markers may be useful in the
differential diagnosis of Alzheimer’s disease and dementia with Lewy bodies. However, this combination of biomarkers did not improve the discrimination of dementia with Lewy bodies or Alzheimer’s disease from either frontotemporal dementia or vascular dementia. Regardless, the study findings indicated that the use of a panel of biomarkers may assist in the distinguishing between different forms of dementia. Additional biomarkers are yet to be identified for the purpose of distinguishing Alzheimer’s dementia from frontotemporal and vascular dementia.

Although stand-alone biomarkers for the diagnosis of an individual neurodegenerative disease are highly desirable, to-date there are no individual stand-alone biomarkers for Alzheimer’s disease. Another aim in Part 2 of this thesis was to develop a means of measuring a potential novel, specific biomarker for Alzheimer’s disease. The identification of potential stand-alone biomarkers often arises from research of literature investigating the underlying mechanisms of individual diseases. It has been shown, for example, that oxidative stress often brings about alterations in the structure and function of proteins that lead to death of the affected cells and initiating the initial pathology of many neurodegenerative diseases. In different neurodegenerative diseases, different proteins are affected by oxidative stress. For example, the protein Pin1 is oxidised in patients with Alzheimer’s disease, but not in patients with other forms of neurodegenerative disease.

We were interested to determine whether measurement of oxidised Pin1 in biological samples might provide a specific and early disease stage biomarker for Alzheimer’s disease. In Chapter 3, we describe the development of an ELISA for measuring oxidised Pin1 in extracts of human brain tissue and used the assay to compare levels of oxPin1 in samples of brain tissue from healthy controls and patients with Alzheimer’s disease. We were able to show that the ratio of oxidised Pin1 to total Pin1 was increased by about 20% in patients with early pathological changes indicative of Alzheimer’s disease. This alludes to the possibility that, with further optimisation of the assay, measurement of oxidised Pin1 in biological fluids such as cerebrospinal fluid may be useful for identifying early pathological changes in the brains of patients with suspected Alzheimer’s disease.

Future development of the ELISA for measurement of oxidised Pin1 in CSF will be required to determine its potential as an indicator of early disease pathology or perhaps as a biomarker of disease.

In Part 3 of the thesis we investigated the use of a panel of biomarkers for the differentiation of Parkinson’s disease from multiple system atrophy, a disease which can resemble Parkinson’s disease at early stages but which progresses much more quickly. Patients with multiple system atrophy may present with specific and sometimes life-threatening symptoms (e.g. nocturnal stridor) which – to some extent – requires a different medical management strategy than Parkinson’s disease. In the first of two studies (Chapter 4), we investigated the combination of two previously established, but not validated, biomarkers, i.e. neurofilament light chain protein and tau protein, together with a more recently identified potential biomarker, FLT3L. We aimed to investigate which of these biomarkers, or combination of biomarkers, might provide maximal discrimination of Parkinson’s disease from multiple system atrophy. We found no evidence to support the use of FLT3L or tau as stand-alone biomarkers for discriminating between multiple system atrophy and Parkinson’s disease. However, neurofilament light chain alone was shown to be useful as a stand-alone biomarker for the discrimination of multiple system atrophy from Parkinson’s disease. Using neurofilament light chain alone we were able
to detect the presence of multiple system atrophy with a sensitivity of 74% and could distinguish multiple system atrophy from Parkinson’s disease with approximately 92% specificity in our patient cohort. Furthermore, we were able to validate these findings in a separate cohort of patients (80% sensitivity and 97% specificity). Combinations of neurofilament light chain and tau, and neurofilament light chain, tau and FLT3L slightly improved the discrimination of multiple system atrophy from Parkinson’s disease but this diagnostic improvement was not statistically significant. Prior to use in clinical practice additional validation studies using neurofilament light chain to discriminate other atypical parkinsonisms from multiple system atrophy and Parkinson’s disease will be required, particularly since levels of neurofilament light chain in Parkinson’s disease did not differ significantly from controls.

In a separate study (Chapter 5), we combined the analysis of DJ-1 protein, known to be altered in patients with genetic forms of the disease, with levels of tau. We were able to demonstrate that DJ-1 was also useful as a stand-alone biomarker for discriminating Parkinson’s disease from controls with high sensitivity (81%) but more moderate specificity (52%). More interestingly, we showed for the first time that levels of DJ-1 were increased by about 70% in multiple system atrophy compared with controls and could distinguish between the two groups with high accuracy (sensitivity of 78% and specificity of 100%). Since DJ-1 levels were also about 35% higher in multiple system atrophy than Parkinson’s disease they were also useful for discriminating between these two groups and the combination of DJ-1 and tau significantly improved the discrimination of multiple system atrophy from Parkinson’s disease (82% sensitivity and 81% specificity). Our observations that DJ-1 is useful in discriminating between Parkinson’s disease and multiple system atrophy, contradicts the findings of other researchers and will require further investigation. This will need to be combined with additional studies enabling validation of the methods used for the measurement of DJ-1 as the assays used in this and other studies have not yet been fully validated for use in CSF.

Using a different approach to biomarker discovery, we also attempted to identify physiological differences between Parkinson’s disease and vascular parkinsonism that might lead to the identification of potential biomarkers for discriminating these disorders. For this purpose, we decided to examine differences in levels of the metabolites of three of the most abundant and physiologically important neurotransmitters. Alterations in neurotransmission, particularly altered levels of the neurotransmitter dopamine, can have significant impact on the initiation of movement and have significant implications in the (patho)physiology of Parkinson’s disease and related disorders. The clinical symptoms of vascular parkinsonism arise due to vascular insults, such as transient ischaemic attacks or cerebrovascular accident (or stroke), which is likely to have a very different effects on neurotransmitter metabolism due to more widespread injury compared with Parkinson’s disease in which degeneration occurs in a very specific subset of neurons. Very few studies have compared differences in neurotransmitter dysfunction in these two disorders. Thus, in Chapter 6 we investigated the levels of several neurotransmitter metabolites to determine whether any differences in levels of these metabolites might indicate potential biomarkers of disease. Although we observed small differences between the two patient groups in their levels of 5-HIAA and HVA, the neurotransmitter metabolites of serotonin and dopamine, respectively, the differences were not sufficiently significant to warrant further investigation of these indices as potential biomarkers. However, our findings were
useful in identifying some possible reasons why patients with vascular parkinsonism generally respond more poorly to the Parkinson’s disease medication, levodopa - used as a dopamine replacement - since the patients with vascular parkinsonism in our study did not have significantly reduced levels of dopamine. This has important implications for management of patients with vascular parkinsonism and warrants further investigation.

In Part 4 of the thesis we developed a method for measuring glutamine synthetase in CSF (Chapter 7) and investigated the potential of glutamine synthetase as a biomarker for the differentiation of neuromyelitis optica from multiple sclerosis (Chapter 8). Since glutamine synthetase is a primarily astrocytic enzyme in the brain, and neuromyelitis optica is a disease involving extensive damage to astrocytes, we hypothesised that we might find increased levels of glutamine synthetase in patients with neuromyelitis optica. Furthermore, since multiple sclerosis is a disease primarily affecting oligodendrocytes rather than astrocytes, we hypothesised that levels of glutamine synthetase would be much greater in neuromyelitis optica than in multiple sclerosis in which we did not expect levels of glutamine synthetase to be altered and might thus be useful in discriminating neuromyelitis optica from multiple sclerosis. We did, indeed find that levels of glutamine synthetase in the CSF of the neuromyelitis patients were approximately double that found for controls and ~22% higher than in multiple sclerosis. Although this effect was greater in patients who tested positive for auto-antibodies directed against the protein aquaporin 4, which regulates the transport of water in astrocytes, levels of glutamine synthetase were also elevated by approximately 69% in multiple sclerosis compared with controls, thus reducing the usefulness of CSF glutamine synthetase as a specific biomarker for neuromyelitis optica.

In general, our studies support the use of CSF biomarkers for the differential diagnosis of neurodegenerative disorders and these findings are discussed in Chapter 9. As with many of the biomarkers already described in the literature, many more studies will be required to confirm the validity of these studies using pathologically confirmed disease and, ultimately, to determine the true utility of these biomarkers in clinical practice.
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Chapter 11

Nederlandse samenvatting

’Spreek in het [Nederlands] als je niet weet hoe iets in het Engels heet, loop met je voeten een ietsje naar buiten en onthoud goed wie je bent!’
Nederlandse samenvatting

Veel neurodegeneratieve ziekten kenmerken zich vaak door heterogene symptomen waardoor het moeilijk kan zijn om neurodegeneratieve ziekten van elkaar te onderscheiden, met name in de beginstadia van deze ziekten. Zoals aangegeven in hoofdstuk 1 (Deel 1 van dit proefschrift) is het ontwikkelen van biomarkers voor de differentiële diagnose van neurodegeneratieve ziekten van uiterst belang voor het optimaliseren van de medische behandeling van patiënten. Momenteel is het onderscheiden van neurodegeneratieve ziekten zeer moeilijk, met name in de beginstadia van de ziekten wanneer het klinische beeld vaak nog niet compleet is. Ook is dan vaak nog niet duidelijk hoe snel een ziekte zich zal ontwikkelen of hoe de reactie op medicatie zal zijn. Daarom is het van belang om nieuwe diagnostische methodes te ontwikkelen die een beter onderscheid mogelijk maken tussen de verschillende ziekten. Het hoofddoel van het onderzoek in dit proefschrift was om de diagnostische potentiële biomarkers te bepalen van een selectie van eiwitten in hersenvocht (cerebrospinale vloeistof, CSF) die eerder beschreven zijn in de literatuur als potentiële biomarkers voor het differentiëren van enkele van de meest bekende vormen van neurodegeneratieve ziekten. Daarnaast was het doel om nieuwe biomarkers al direct bruikbaar te maken bij het onderscheiden van neurodegeneratieve ziekten met vergelijkbare symptomen op het moment van de CSF opname. In dit onderzoek hebben we naast elkaar onderzocht of deze biomarkers al direct bruikbaar waren bij het onderscheiden van verschillende vormen van dementie (Deel 2), bewegingsstoornissen (Deel 3) en demyeliniserende aandoeningen (Deel 4).

In deel 2 van dit proefschrift is aandacht besteed aan de verbetering van differentiële diagnose van dementie. In eerste instantie hebben we gekeken naar mogelijkheden om de traditionele biomarkers voor de ziekte van Alzheimer te verbeteren door het toevoegen van een extra biomarker parameter. In een eerdere studie is bewezen dat het toevoegen van 3-methoxy-4-hydroxyphenylglycol (MHPG; de metaboliet van de neurotransmitter epinefrine) aan de traditionele Alzheimer biomarkers kan helpen in het differentiëren van de ziekte van Alzheimer en dementie met Lewy lichaampjes. In hoofdstuk 2 hebben we onderzocht of we dit ook konden bevestigen in een onafhankelijke groep patiënten en of deze combinatie van eiwitten ook een beter onderscheid kon geven voor dementie met Lewy lichaampjes en vasculaire of frontotemporale dementie. Deze biomarker combinatie bleek inderdaad een beter onderscheid te geven tussen de ziekte van
Alzheimer en dementie met Lewy lichaampjes dan de traditionele Alzheimer biomarkers alleen. De detectiegevoeligheid van de ziekte van Alzheimer was door het toevoegen van MHPG aan de traditionele biomarkers ook met een lichte stijging in sensitiviteit van 62% naar 65% verbeterd en deze combinatie vormt daardoor een mogelijk nuttige biomarker voor de differentiële diagnose tussen de ziekte van Alzheimer en dementie met Lewy lichaampjes. Deze biomarker combinatie gaf echter geen verbetering in het kunnen onderscheiden tussen dementie met Lewy lichaampjes en vasculaire of frontotemporale dementie. Desondanks is het gebruik van een panel biomarkers voor het onderscheiden van verschillende vormen van dementie door deze resultaten wel aangetoond. Wij concluderen dat toevoeging van nog meer biomarkers noodzakelijk zijn voor de differentiatie tussen de ziekte van Alzheimer en/of dementie met Lewy lichaampjes en vasculaire en frontotemporale dementie.

Hoewel zelfstandige biomarkers zeer gewenst zijn voor de diagnose van individuele neurodegeneratieve ziekten, zijn er tot nu toe nog geen zelfstandige biomarkers voor de ziekte van Alzheimer. Een ander doel van dit proefschrift was de ontwikkeling van een nieuwe en specifieke diagnostische test voor de ziekte van Alzheimer gebaseerd op het meten van slechts één eiwit in CSF. Het onderzoek naar potentieel zelfstandige biomarkers is gebaseerd op eerder, in vakliteratuur beschreven, onderzoek naar onderliggende mechanismen van individuele ziekten. Het is bijvoorbeeld bekend dat oxidatieve stress een rol kan spelen in ongewenste wijzigingen in de structuur en de functie van eiwitten, dat kan leiden tot het afsterven van cellen en de pathologie van veel neurodegeneratieve ziekten kan initiëren. In een neurodegeneratieve ziekte kunnen specifieke, verschillende eiwitten aangetast zijn door oxidatieve stress. Het eiwit Pin1 bijvoorbeeld is geoxideerd bij patiënten met de ziekte van Alzheimer maar niet bij patiënten met andere neurodegeneratieve ziekten. Wij waren geïnteresseerd of geoxideerd Pin1 in biologisch materiaal een specifieke biomarker voor de ziekte van Alzheimer kan zijn, met name in vroege stadia van de ziekte. In hoofdstuk 3 hebben we de ontwikkeling van een nieuwe ELISA methode voor het meten van geoxideerd Pin1 beschreven. We hebben dit eiwit in hersenweefsel van gezonde controles en van patiënten met de ziekte van Alzheimer gemeten en we tonen aan dat de ratio tussen geoxideerd Pin1 en de totale hoeveelheid Pin1 verhoogd is in ongeveer 20% van de patiënten met Alzheimer type pathologische aandoeningen in hun hersenen. De resultaten wijzen op de mogelijkheid dat, met verder optimaliseren van de ELISA, het meten van geoxideerd Pin1 in CSF mogelijk nuttig kan zijn voor het vroege ontdekken van pathologische veranderingen in de hersenen van patiënten met Alzheimer-type dementie. Er is nader onderzoek nodig om definitief vast te stellen of het meten van geoxideerd Pin1 in CSF potentie heeft om te dienen als biomarker voor beginnende Alzheimer dementie.

In Deel 3 van dit proefschrift hebben we het gebruik onderzocht van een panel biomarkers voor het differentiëren tussen de ziekte van Parkinson en multiple systeem atrofie, een ziekte die heel erg op de ziekte van Parkinson lijkt maar een versneld ziektebeeld kent. Patiënten met multiple systeem atrofie hebben soms symptomen die levensgevaarlijk kunnen zijn (bijvoorbeeld nachtelijke stridor) waarvoor een andere medische behandeling nodig is dan die voor de ziekte van Parkinson. In de eerste van twee studies (Hoofdstuk 4) hebben we de combinatie van twee vastgestelde maar nog niet gevalideerde biomarkers (neurofilament light chain eiwit en tau eiwit) samen met een meer recentelijk ontdekte potentiële biomarker (FLT3L eiwit) onderzocht. Ons doel was te ontdekken welke van
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dezo biomarkers, of combinatie van biomarkers, de beste discriminatie geeft tussen de ziekte van Parkinson en multiple systeem atrofie. Onze resultaten laten zien dat het FLT3L eiwit en het tau eiwit hiervoor niet als zelfstandige biomarkers gebruikt kunnen worden, maar het neurofilament light chain eiwit wel. Bij het gebruik van alleen neurofilament light chain konden we in onze patiëntengroepen met een sensitiviteit van ongeveer 74% multiple systeem atrofie detecteren en konden we met een specificiteit van 92% het onderscheid maken tussen multiple systeem atrofie en de ziekte van Parkinson. Bovendien was het mogelijk om deze resultaten te valideren in een tweede groep van patiënten (met 80% sensitiviteit en 97% specificiteit). De combinaties van neurofilament light chain met tau en van neurofilament light chain met tau en FLT3L gaf een kleine verbetering in deze resultaten, maar de verschillen waren statistisch niet significant. Omdat de concentratie van neurofilament light chain niet significant verschillend was tussen gezonde controles en mensen met de ziekte van Parkinson dient, vóór de toepassing van deze biomarker(s) in de klinische praktijk, het gebruik van neurofilament light chain voor het onderscheiden van andere atypische parkinsonismen en multiple systeem atrofie verder onderzocht worden.

In een tweede studie (hoofdstuk 5), hebben we een analyse uitgevoerd van het DJ-1 eiwit, waarvan bekend is dat deze wordt aangetast in genetische vormen van de ziekte van Parkinson, in combinatie met het eiwit tau. We laten zien dat DJ-1, als zelfstandige biomarker, met een hoge sensitiviteit (81%), maar een gemiddelde specificiteit (52%), het onderscheid kan maken tussen de ziekte van Parkinson en gezonde controles. Bovendien hebben we ontdekt dat concentraties van DJ-1 in CSF zijn verhoogd met ongeveer 70% bij de patiënten met multiple systeem atrofie in vergelijking met controles. De biomarker DJ-1 maakt met hoge accuratesse (sensitiviteit 78% en specificiteit 100%) onderscheid tussen deze twee groepen. Omdat ook de concentratie van DJ-1 bij multiple systeem atrofie in vergelijking met de ziekte van Parkinson ongeveer 35% hoger ligt is DJ-1, in combinatie met tau, ook nuttig voor de discriminatie tussen de ziekte van Parkinson en multiple systeem atrofie (sensitiviteit 82% en specificiteit 81%). Deze bevindingen zijn tegenstrijdig met eerdere bevindingen van andere onderzoekers, en verder onderzoek is dus noodzakelijk om onze resultaten te valideren. Ook de DJ-1 ELISA die in dit onderzoek was gebruikt dient nog nader onderzocht te worden omdat deze nog niet volledig is gevalideerd voor meting in CSF.

We hebben een andere benadering gebruikt om fysiologische verschillen tussen de ziekte van Parkinson en vasculair parkinsonisme te bestuderen die als mogelijke biomarkers kunnen fungeren voor de discriminatie tussen deze ziekten. We hebben hiervoor concentraties van drie van de meest voorkomende en belangrijke neurotransmitter metabolieten in CSF onderzocht. Veranderingen in neurotransmissie, met name gewijzigde concentraties van dopamine, kunnen grote invloed hebben op het initiëren van beweging en kunnen grote gevolgen hebben in de (patho)fysiologie van de ziekte van Parkinson en daaraan gerelateerde bewegingsstoornissen. Het klinische beeld van vasculair parkinsonisme wordt door cerebrovasculaire beschadiging zoals een cerebrovascular accident (CVA of hersenbloeding) of een transiënte ischemische aanval (TIA) veroorzaakt. Dit heeft waarschijnlijk een heel ander effect op het neurotransmitter metabolisme als gevolg van meer wijdverbreide schade in vergelijking met de ziekte van Parkinson, waarin juist degeneratie van een zeer specifieke subset van neuronen wordt aangetroffen. Weinig studies hebben de verschillen in neurotransmitter dysfunctie tussen
deze ziekten vergeleken. Daarom hebben we in hoofdstuk 6 onderzocht of verschillen in neurotransmitter metaboliet concentraties in CSF als biomarkers kunnen dienen voor het onderscheid tussen de ziekte van Parkinson en vasculair parkinsonisme. Hoewel er kleine verschillen in 5-HIAA en HVA (de respectievelijke metabolieten van serotonine en dopamine) gevonden werden tijdens deze twee patiëntengroepen bleken deze verschillen niet significant genoeg te zijn om deze metabolieten verder te onderzoeken als potentiële biomarkers. Onze bevindingen geven wel mogelijke verklaringen waarom het Parkinson medicijn levodopa – dat als vervanging dient voor het tekort aan dopamine – niet goed werkt bij patiënten met vasculair parkinsonisme. Uit onze resultaten blijkt namelijk dat de patiënten met vasculair parkinsonisme in onze studie geen gebrek aan dopamine hadden. Dit impliceert dat verder onderzoek benodigd is voor de behandeling van patiënten met vasculair parkinsonisme.

In Deel 4 van dit proefschrift hebben we een door ons ontwikkelde methode beschreven voor het meten van het eiwit glutamine synthetase in CSF (Hoofdstuk 7). Met gebruik van deze methode hebben we vervolgens onderzocht of glutamine synthetase een mogelijke biomarker kan zijn voor het onderscheiden van neuromyelitis optica en multiple sclerose (Hoofdstuk 8). Omdat glutamine synthetase voornamelijk een astrocytair enzym is en neuromyelitis optica een ziekte met veel astrocytaire schade is, hebben we een hypothese opgesteld waarin glutamine synthetase in CSF mogelijk toegenomen is bij mensen met neuromyelitis optica. Daarnaast hebben we een hypothese opgesteld waarin glutamine synthetase meer is toegenomen in CSF van neuromyelitis optica patiënten dan in CSF van multiple sclerose patiënten omdat bij multiple sclerose voornamelijk oligodendrocyten in plaats van astrocyten aangedaan zijn. Onze verwachting was dat de concentraties van glutamine synthetase veel hoger zouden zijn in neuromyelitis optica dan in multiple sclerose en dat dit eiwit daarom een mogelijke biomarker zou zijn voor het differentiëren tussen deze ziekten. Ons onderzoek liet zien dat glutamine synthetase inderdaad bijna twee keer zo hoog was bij onze neuromyelitis optica patiënten dan in controles en ook 22% hoger dan bij multiple sclerose patiënten. Hoewel dit effect groter was bij patiënten waarbij auto-antilichamen zijn aangetroffen gericht tegen het eiwit aquaporine 4 (dat een rol speelt in het transport van water in astrocyten) lieten de resultaten tevens zien dat glutamine synthetase ook ongeveer 68% hoger was bij patiënten met multiple sclerose in vergelijking met controles. Uit deze resultaten blijkt dat het verschil in concentraties van glutamine synthetase tussen neuromyelitis optica en multiple sclerose niet groot genoeg is om glutamine synthetase als biomarker te gebruiken.

Over het algemeen ondersteunen onze studies het gebruik van CSF biomarkers voor de differentiële diagnose van neurodegeneratieve ziekten en deze bevindingen worden bediscussieerd in Hoofdstuk 9.

Tot slot: Zoals ook voor vele eerder beschreven potentiële biomarkers dient er meer onderzoek gedaan te worden met biologisch materiaal van patiënten met pathologisch bewezen ziekte om te bewijzen dat deze eiwitten als goede biomarkers kunnen dienen en wat deze biomarkers uiteindelijk kunnen toevoegen in de klinische praktijk.
Acknowledgements/Dankwoord
Curriculum vitae and publications
Publication Series

"Oh my ears and whiskers, how late it's getting!"
Acknowledgements/Dankwoord

“One of the deep secrets of life is that all that is really worth the doing is what we do for others.”

“And what is the use of a book,” thought Alice, “without pictures or conversation?”
Dankwoord
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I've always been a square peg trying to fit into a round hole. Never more so than making the move to the Netherlands where I've had to fit in to a new (albeit not-so-different) culture, and learn the nuances of a new language. Nonetheless, I found my way into a job as a Research Assistant in the Neurology Department at the Radboud UMC and, a year later, as a PhD student. Despite all my efforts to fit in, to 'round off the edges' so to speak, I'm still that same square peg with, perhaps, slightly softened edges. However, I've learned one simple but important thing: I am who I am and most people just accept that. Not to forget, who I am has written this thesis. But not without help and there will never be a more perfect opportunity to reflect on the people, places and events that helped me get to this point.

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In the final stages of a Dutch PhD comes the need to compile all the papers into a final, presentable book. I am eternally grateful to Petra Oolders for creating the fabulous photos that appear throughout my thesis. She managed to bring the ideas in my head to life. I am indebted to her for helping to make my thesis come alive. I must also thank Ineke for being the face of my thesis and for allowing me to present her story as an example of the importance of scientific research in this field. Ook wil ik Ineke en Sofie bedanken omdat zij model hebben gestaan voor de foto’s.

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(In Dunglish, onze taal) Lieve, lieve Jan, mijn tegenpool; you bring calm to my chaos, tranquility to my endless chatter, a stroll to my run, moderation to my impatience. Samen kunnen we alles.
Curriculum vitae and publications
Megan Herbert

“Be what you would seem to be - or, if you’d like it put more simply - never imagine yourself not to be otherwise than what it might appear to others that what you were or might have been was not otherwise than what you had been would have appeared to them to be otherwise.”
Curriculum Vitae

Megan was born on the 21st of October, 1968 in Wallaroo, South Australia. In 1986 she completed her high school diploma in Adelaide and began work as a laboratory technician at the Queen Elizabeth Hospital. In 1997, while working at the Queen Elizabeth Hospital, she started an Honours degree in Applied Science (Speech Pathology), graduating in 2001 with the Speech Pathology Australia prize for outstanding academic achievement. After working for 2 years as a Speech Pathologist (logopedist), she returned to the laboratory as a Research Assistant.

In her first appointment as Research Assistant in the Sleep Laboratory at the Royal Adelaide Hospital, under the supervision of Dr Ral Antic and Dr Andrew Thornton, she investigated the link between sleep apnoea and high blood pressure in patients with kidney disease. In her next appointment in the Clinical Pharmacology laboratory of the Queen Elizabeth Hospital together with Dr Betty Sallustio, Prof Dr John Horowitz and Dr Ray Morrison, she investigated the clinical efficacy of the enantiomers of Perhexiline, a drug prescribed for the treatment of angina in patients who fail to respond to other medications. Shortly after completing this project she worked on a project lead by Dr Hakan Muyderman and Dr Neil Sims to develop methods for downregulating the GPx-1 gene in cultured rat astrocytes using siRNA in order to ultimately look at new treatments for stroke. Concurrently with the last two projects (2004-2006), she undertook a double Bachelor degree in Medical Science and Biotechnology (Forensics) at the Charles Sturt University completing her last year by distance education following her move to the Netherlands. She graduated with Distinction in 2009 receiving a Deans award for Academic Excellence. In the same year Megan began work as a Research Assistant in the Neurochemistry Laboratory at the Radboud University Medical Centre in Nijmegen and was appointed one year later as a PhD student in the same laboratory. In this position she was supervised by Dr Bea Kuiperj, Dr Marcel Verbeek and Prof Dr Bas Bloem.

The research described in this thesis investigated the use of CSF biomarkers for the differential diagnosis of a variety of neurodegenerative diseases including Alzheimer’s dementia, Parkinson’s disease, and multiple sclerosis. This resulted in several publications in scientific journals. Since 2013 Megan has been working as a post-doctoral researcher in the Biomolecular Chemistry laboratory at the Radboud University on a project investigating the role of autoantibodies in sporadic inclusion body myositis.
Publications of Megan Herbert

Original Papers

Herbert MK, Aerts MB, Beenes MB, Norgren N, Esselink R, Bloem BR, Kuiperij HB, Verbeek MM. Combined cerebrospinal fluid levels of neurofilament light chain, FLT3L and total tau distinguish multiple system atrophy from Parkinson’s disease. [Submitted]


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“The time has come,” the walrus said, “to talk of many things: Of shoes and ships - and sealing wax - of cabbages and kings”
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12. Wandana Nanhoe-Mahabier. Freezing and falling in Parkinson's disease: from the laboratory to the clinic. Radboud University Nijmegen, 13 February 2012
13. Marlies van Nimwegen. Promotion of physical activity in Parkinson's disease, the challenge to change behavior. Radboud University Nijmegen, 6 March 2013
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“Alice had got so much into the way of expecting nothing but out-of-the-way things to happen, that it seemed quite dull and stupid for life to go on in the common way.”